

**POLYMORPHIC ELEMENTS IN THE COSTIMULATORY RECEPTOR  
LOCUS AND USES THEREOF**

**5     *Cross-Reference to Related Applications***

This application claims priority to provisional application serial number 60/126,215, entitled "Polymorphism of CTLA-4 and Uses Thereof," filed on March 25, 1999. This application is a continuation-in-part of USSN 09/534,061, filed on March 24, 2000, which corresponds to International Application Serial No.

- 10   PCT/US00/07938 (Publication No. WO 00/56856) filed March 24, 2000. The entire contents of these applications are incorporated herein by reference. Attached hereto is Appendix A containing materials related to this application. The entire contents of this appendix is hereby incorporated by reference.

**15    *Background of the Invention***

- In order for T cells to respond to foreign proteins, two signals must be provided by antigen-presenting cells (APCs) to resting T lymphocytes (Jenkins, M. and Schwartz, R. (1987) *J. Exp. Med.* 165, 302-319; Mueller, D.L., et al. (1990) *J. Immunol.* 144, 3701-3709). The first signal, which confers specificity to the immune response, is transduced via the T cell receptor (TCR) following recognition of foreign antigenic peptide presented in the context of the major histocompatibility complex (MHC). The second signal, termed costimulation, induces T cells to proliferate and become functional (Lenschow et al. 1996. *Annu. Rev. Immunol.* 14:233). Costimulation is neither antigen-specific, nor MHC restricted and is thought to be provided by one or more distinct cell surface molecules expressed by APCs (Jenkins, M.K., et al. 1988 *J. Immunol.* 140, 3324-3330; Linsley, P.S., et al. 1991 *J. Exp. Med.* 173, 721-730; Gimmi, C.D., et al., 1991 *Proc. Natl. Acad. Sci. USA.* 88, 6575-6579; Young, J.W., et al. 1992 *J. Clin. Invest.* 90, 229-237; Koulova, L., et al. 1991 *J. Exp. Med.* 173, 759-762; Reiser, H., et al. 1992 *Proc. Natl. Acad. Sci. USA.* 89, 271-275; van-Seventer, G.A., et al. (1990) *J. Immunol.* 144, 4579-4586; LaSalle, J.M., et al., 1991 *J. Immunol.* 147, 774-80; Dustin, M.I., et al., 1989 *J. Exp. Med.* 169, 503; Armitage, R.J., et al. 1992 *Nature* 357, 80-82; Liu, Y., et al. 1992 *J. Exp. Med.* 175, 437-445).

The CD80 (B7-1) and CD86 (B7) proteins, expressed on APCs, are critical costimulatory molecules (Freeman et al. 1991. *J. Exp. Med.* 174:625; Freeman et al. 1989 *J. Immunol.* 143:2714; Azuma et al. 1993 *Nature* 366:76; Freeman et al. 1993. *Science* 262:909). B7 appears to play a predominant role during  
5 primary immune responses, while B7-1, which is upregulated later in the course of an immune response, may be important in prolonging primary T cell responses or costimulating secondary T cell responses (Bluestone. 1995. *Immunity*. 2:555).

One receptor to which B7-1 and B7 bind, CD28, is constitutively expressed on resting T cells and increases in expression after activation. After  
10 signaling through the T cell receptor, ligation of CD28 and transduction of a costimulatory signal induces T cells to proliferate and secrete IL-2 (Linsley, P.S., et al. 1991 *J. Exp. Med.* 173, 721-730; Gimmi, C.D., et al. 1991 *Proc. Natl. Acad. Sci. USA*. 88, 6575-6579; June, C.H., et al. 1990 *Immunol. Today*. 11, 211-6; Harding, F.A., et al. 1992 *Nature*. 356, 607-609). A second receptor, termed CTLA4 (CD152)  
15 is homologous to CD28 but is not expressed on resting T cells and appears following T cell activation (Brunet, J.F., et al., 1987 *Nature* 328, 267-270). CTLA4 appears to be critical in negative regulation of T cell responses (Waterhouse et al. 1995. *Science* 270:985). Blockade of CTLA4 has been found to remove inhibitory signals, while aggregation of CTLA4 has been found to provide inhibitory signals that downregulate  
20 T cell responses (Allison and Krummel. 1995. *Science* 270:932). In addition, lymphoproliferative disease has been associated with CTLA-4 gene-deficient mice (Bluestone, J.A., et al. (1997). *J. Immunol* 158: 1989-93; June et al., (1994) *Immunol Today* 15: 321-31; Tivol et al., (1996). *Curr Opin Immunol* 8:822-30; Tivol et al. (1995) *Immunity* 3: 541-7), although data conflicting this interpretation also exist  
25 (Liu, Y. (1997). *Immunol Today* 18: 569-72; Wu, Y. et al. (1997) *J Exp Med* 185: 1327-35; Zheng, Y., et al. (1998) *Proc Natl Acad Sci USA* 95: 6284-9). Recently, a CD28-like receptor ICOS (Hutloff et al. 1999) and its B7-like cognate ligand, GL50 was identified in both mouse and humans systems (Ling et al. 2000 *J Immunol* 164: 1653-7; also known as B7RP or B7h, Yoshinaga, S. K., et al. 1999. *Nature* 402: 827;  
30 Swallow, M. M., et al. 1999 *Immunity* 11: 423). CD28 and ICOS exhibit protein sequence identity of ~24 %, just as the GL50 proteins also share ~24% sequence identity with B7 proteins. Despite structural similarity, neither GL50 nor ICOS are likely to utilize the B7:CD28/CTLA4 costimulatory pathways because of the inability

of GL50 to bind CD28/CTLA4 proteins and of the inability of B7 proteins to bind ICOS receptors (Ling, V., et al. 1999. *Genomics* 60: 341). In vitro analysis of ICOS mediated T-cell costimulation revealed that ICOS engagement resulted in enhanced T cell proliferation and Th-2 cytokine production. Blockade of the ICOS pathway by addition of ICOS-Ig to MLR (mixed lymphocyte reaction) or tetanus toxoid recall response assays resulted in decreased T-cell proliferation (Aicher, A., et al. 2000. *J Immunol* 164: 4689-96.). Transgenic mice expressing ICOS-ligand exhibited an increase in B-cell germinal center size and enhancement of immunoglobulin production (Yoshinaga *et al.*, supra) suggesting that overexpression of the ligand may influence B cell development. Taken together, these data are consistent with the model of the ICOS receptor serving as a pivotal signaling molecule involved with T-cell and B-cell proliferation and differentiation.

The genetic organization of CTLA-4 has been previously described (Brunet, J. F., *et al.*, (1987). *Nature* 328: 267-70; Dariavach, P., *et al.*, (1988). *Eur J Immunol* 18: 1901-5.) as being comprised of 4 exons which encode separate functional domains: a leader sequence, an extracellular domain, a transmembrane domain, and cytoplasmic domain. Within the extracellular domain, the B7 binding motif is centered on the amino acids MYPPPY, a sequence also found in the extracellular domain of CD28, the primary B7 receptor responsible for T-cell activation (Balzano, C., *et al.*, (1992). *Int J Cancer Suppl* 7: 28-32). The cytoplasmic domain of CTLA-4 encodes the motif YVKM in which the phosphorylation state of tyrosine has been implicated in both signal transduction through SYP/SHP2 phosphatase (Marengere, L. E., *et al.*, (1996). *Science* 272: 1170-3. [published errata appear in *Science* 1996 Dec 6;274(5293)1597 and 1997 Apr 4;276(5309):21]; Shiratori, T., *et al* (1997). *Immunity* 6: 583-9), and the intracellular accumulation of CTLA-4 via AP50 clathrin-mediated endocytosis (Chuang, E., *et al.*, (1997). *J Immunol* 159: 144-51; Zhang, Y., and Allison, J. P. (1997) *Proc Natl Acad Sci U S A* 94: 9273-8). CTLA-4 has also been reported to be involved with T-cell receptor signaling by interfering with ERK and JNK activation (Calvo, C. R., *et al.*, (1997). *J Exp Med* 186: 1645-53). Recently, polymorphisms in the non-coding region 3' of human CTLA-4 DNA have been correlated with a number of autoimmune diseases, including: Grave's disease (Donner, H., *et al.*, (1997a). *J Clin Endocrinol Metab* 82: 4130-2 Donner, H., *et al.*, (1997b). *J Clin Endocrinol Metab* 82: 143-6; Kotsa, K., *et*

- al., (1997). *Clin Endocrinol (Oxf)* 46: 551-4; Nistico, L., et al., (1996). *Hum Mol Genet* 5: 1075-80), Hashimoto's disease (Braun, J., et al., (1998). *Tissue Antigens* 51: 563-6; Tomer, Y., et al., (1997). *J Clin Endocrinol Metab* 82: 1645-8, myasthenia gravis with thymoma (Huang, D., et al., (1998). *J Neuroimmunol* 88: 192-8), and
- 5 IDDM (Marron, M. P., et al., (1997). *Hum Mol Genet* 6: 1275-82; Nistico, L., et al., (1996). *Hum Mol Genet* 5: 1075-80) in patients.

The minimal promoter of mouse CTLA-4 suggests that transcriptional initiation control is localized approximately 335 bp upstream from the initiation codon. However, the contribution from other regions of the CTLA-4 locus to the

10 regulation of gene expression has not been examined (Finn, P. W., et al., (1997). *J Immunol* 158: 4074-81; Perkins, D., et al., (1996). *J Immunol* 156: 4154-9). Despite the tightly regulated control of CTLA-4 expression and the importance of this key immunoregulatory protein, the published genomic sequences of the human CTLA-4 are incomplete. Further, no data are available for the intron sequences of mouse

15 CTLA-4. In addition, the genomic structure of other costimulatory receptors is not well understood.

Areas of simple repetitive DNA (i.e., microsatellite DNA) interspersed throughout the genome have been used extensively to map chromosomes. It has been found that these simple repeats often vary in length among individuals, thus, they

20 have facilitated genetic linkage studies of diseases within populations. Unlike long and short interspersed repeats, the mechanism by which simple repeats are generated and inserted into the genome is not known, and their potential role in modulating biochemical processes is not clear (Epplen, C., et al., (1997). *Electrophoresis* 18: 1577-85; Epplen, J. T., et al., (1994). *Biol Chem Hoppe Seyler* 375: 795-801). In

25 addition, single nucleotide polymorphisms (SNPs), resulting from variations, insertions, or deletions, result in base changes that contribute to the majority of phenotypic diversity.

Certain polymorphisms of a particular sequence in particular regions have been correlated with the development of, or susceptibility, to a disease or other

30 condition. Because the genes responsible for disorders or conditions associated with the immune response have not all been cloned, it is useful to utilize such markers for a variety of diagnostic and prognostic assays. The utility of such markers depends upon how tightly the marker and the disease locus are linked. Accordingly, the

identification of novel DNA polymorphisms that are associated with disease states is desirable and aids in the diagnosis or prognosis of diseases or conditions to which they are linked.

## 5 *Summary of the Invention*

This application relates, at least in part, to the identification of polymorphic elements, such as microsatellite repeat ("PMR") or single nucleotide polymorphisms ("SNP") sequences in the costimulatory receptor gene locus. These sequences are useful as markers e.g., identifying genetic material from a given individual and/or in identifying individuals at risk for developing a particular disease or condition or at risk for giving birth to an offspring likely to develop a particular disease or condition. In particular, the subject markers are linked to a variety of autoimmune diseases or conditions.

In one aspect, the invention pertains to a method for determining the predisposition of a human subject to develop autoimmune disease, said method comprising detecting a polymorphic microsatellite repeat (PMR) in the human costimulatory receptor gene locus, wherein the PMR sequence is not an hR2 sequence, to thereby determine the predisposition of a human subject to develop autoimmune disease.

In one embodiment, the PMR sequence selected from the group consisting of SEQ ID Nos.: 303, 306, 309, 312, 315, 321, 324, 327, 330, 333, 336, 339, 342, 345, 348, 351, 354, 357, 360, 363, 366, and 369.

In another embodiment, the autoimmune disease is selected from the group consisting of: insulin-dependent diabetes mellitus (IDDM), Addison's disease, Graves' disease, autoimmune hypothyroidism, myasthenia gravis, thymoma, lupus, thyroiditis, postpartum thyroiditis, rheumatoid arthritis, Hashimoto's disease, coeliac disease and leprosy.

In one embodiment, the step of detecting is performed using a polymerase chain reaction (PCR) employing a first and second primer.

In one embodiment, the first or second comprises the sequence selected from the group consisting of SEQ ID Nos.: 301, 302, 304, 305, 307, 308, 310, 311, 313, 314, 316, 317, 319, 320, 322, 323, 325, 326, 328, 329, 331, 332, 334, 335, 337,

338, 340, 341, 343, 344, 346, 347, 349, 350, 352, 353, 355, 356, 358, 359, 361, 362, 364, 365, 367, and 368.

In another aspect, the invention pertains to a method for determining the predisposition of a human subject to autoimmune disease, said method comprising  
5 detecting an hR1 PMR sequence to thereby determine the predisposition of a human subject to autoimmune disease.

In one embodiment, the autoimmune disease is selected from the group consisting of insulin-dependent diabetes mellitus (IDDM), Addison's disease, Graves' disease, autoimmune hypothyroidism, myasthenia gravis, thymoma, lupus, thyroiditis,  
10 postpartum thyroiditis, rheumatoid arthritis, Hashimoto's disease, coeliac disease and leprosy.

In one embodiment, the step of detecting is performed using PCR employing a first and second primer.

In another aspect, the invention pertains to a method for determining  
15 the polymorphic variant or subtype of a PMR sequence in the costimulatory receptor locus in a human subject, said method comprising detecting a polymorphic microsatellite repeat (PMR) in the human costimulatory receptor gene locus, wherein the PMR sequence is not an hR2 sequence to thereby determine the polymorphic variant or subtype of a PMR sequence in the costimulatory receptor locus in a human  
20 subject.

In one embodiment, the PMR sequence is selected from the group consisting of SEQ ID Nos.: 3, 6, 9, 12, 15, 18, 21, 24, 27, 30, 33, 36, 39, 42, 44, 48, 51, 54, 57, 60, 63, 66, 69, 72, 75, 78, 81, 84, 87, 90, 93, 96, 99, 102, 105, 108, 111, 114, 117, 120, 123, 126, 129, 132, 135, 138, 141, 144, 147, 150, 153, 156, 159, 162,  
25 165, 168, 171, 174, 177, 180, 183, 186, 189, 192, 195, 198, 201, 204, 207, 210, 213, 216, 219, 222, 225, 228, 231, 234, 237, 240, 243, 246, 249, 252, 255, 258, 261, 264, 267, 270, 273, 276, 279, 282, 285, 288, 291, 294, 297, and 300.

In one embodiment, the step of detecting is performed using PCR employing a first and second primer.

In another aspect, the invention pertains to a PCR primer capable of  
30 amplifying a PMR sequence in the costimulatory receptor locus of a human subject, wherein the primer consists of a nucleotide sequence selected from the group consisting of: SEQ ID NO: 301, 302, 304, 305, 307, 308, 310, 311, 313, 314, 316,

317, 319, 320, 322, 323, 325, 326, 328, 329, 331, 332, 334, 335, 337, 338, 340, 341, 343, 344, 346, 347, 349, 350, 352, 353, 355, 356, 358, 359, 361, 362, 364, 365, 367, and 368.

In still another aspect, the invention pertains to a method for  
5 determining the predisposition of a human subject to develop autoimmune disease, said method comprising detecting single nucleotide polymorphism (SNP) in the human costimulatory receptor gene, to thereby determine the predisposition of a human subject to develop autoimmune disease.

## 10 **Brief Description of the Drawings**

Figure 1 is a sequence diagram of the human 2q33 costimulatory receptor region. The position of sequence line is indicated as nt. displayed. The  
15 stippled line represents human BAC clone 22700 sequence. Coding sequences of NADH:ubiquinone oxidoreductase, keratin-18 pseudogene, and nucleophosmin pseudogene, EST-like sequences, retroviral elements, CD28 (4 CDS), CTLA4 (4 CDS) and the ICOS (5 CDS) receptors are displayed as open boxes on the sequence  
20 line. Black bars beneath sequence line indicate regions of mouse sequence homology (>35 bp, >70% identity) based on limited sequencing of mouse BAC clone 23114 syntenic to human BAC clone 22700. White boxes below the sequence line indicate predicted ORFs by Grail; gray boxes indicate predicted ORFs by DiCTion. Sequences with homologies to Genbank STS and microsatellite repeats are marked as  
25 asterisks. Several of the polymorphic microsatellite repeats used in this study are indicated as SARA 43, SARA 1, SARA 31, CTLA4 3' UTR, and SARA 47, referring to the first primer of the primer pair used to amplify them.

Figure 2 panels A and B show hybridization analysis of 2q33  
25 sequences. Panel A shows results of genomic microarray expression analysis of BAC clone 22700 sequences. Inserts from the sequenced BAC clone 22700 library were amplified and spotted onto glass slides. RNA probes were generated from either non-induced or PMA-ionomycin induced human CD4+ T-cells. Differential hybridization  
30 in 5/6 experiments yielded clones corresponding to those positions presented. Panel B shows identification of anti-sense ICOS transcripts. RNA blot of activated and non-activated RNA samples from two donor CD4+ T-cells preparation and Jurkat cell line were hybridized against strand-specific (either + or -) radiolabeled T7-transcripts of

ICOS 3'-UTR region (right line drawing). ICOS 3'-UTR (-) probe hybridization reveals ICOS gene transcripts (left blot) while ICOS 3' UTR (+) probe hybridization reveals LTR derived anti-sense-ICOS transcripts (right blot).

Figure 3 shows identification of polymorphic microsatellite repeats within BAC clone 22700. Amplification of repeats amplified by SARA 31, CTLA4 3' UTR, SARA 1, SARA 43, and SARA 47 followed by denaturing PAGE electrophoresis and autoradiography revealed polymorphic PCR products. Two alleles were detected in SARA 31 and CTLA4 3' UTR; 4 alleles were detected in SARA 1, and >5 alleles were detected in both SARA 43 and SARA 47 amplification reactions.

Figure 4 panels A, B, and C, show sequence alignment between mouse and human ICOS genomic DNA. Panel A shows GAP alignment of regions flanking CDS-1 (boxed) revealed two zones of sequence homology (as shown) separated by a ~250 bp mouse-specific repetitive DNA region. Panel B shows dot plot alignment of human and mouse ICOS genomic regions including CDS-2 to CDS-5. Homologies greater than 60% identity over a 20 bp window are displayed. Panel C shows similarity plot of consensus sequence derived from GAP alignment between human and mouse ICOS genomic regions displayed in B. Breaks in similarity index indicates presence of non-conserved repetitive sequences. Aligned consensus coding sequences are indicated in top line while location of the conserved microsatellite repeat amplified by the SARA 47 primer set is denoted by an asterisk.

#### ***Detailed Description of the Invention***

The instant invention provides polymorphic elements, e.g., polymorphic microsatellite repeat ("PMR") or single nucleotide polymorphism ("SNP") sequences in the costimulatory receptor gene locus. The invention also provides sequences that can be used to amplify PMR or SNP sequences. The polymorphic elements of the invention are useful as markers e.g., in genetic testing, for example, to identify genetic material from a given individual and/or in identifying individuals at risk for developing a particular disease or condition. In particular, the subject polymorphic elements are useful in identifying individuals that carry or are at risk for developing diseases or conditions associated with signaling via a costimulatory receptor, such as CD28, CTLA4, or ICOS, e.g., autoimmune diseases or



conditions. Tables I and II list the sequences of PMRs of the invention and Table III lists the sequences comprising the SNPs of the invention (the SNP is shown in a bold uppercase letter).

## 5 I. Definitions

As used herein the term "costimulatory receptor gene locus" includes the genetic region comprising the genes encoding the costimulatory receptors CD28, CTLA4, and ICOS. This locus spans approximately 300 kb on chromosome 2q33.

As used herein the term "polymorphic microsatellite repeat (PMR)"  
10 includes regions of a chromosome containing runs of short repeated sequences (e.g., ATATAT). These simple microsatellite DNA repeats tend to be interspersed throughout the genome and the number of such repeats is highly variable in the population. For example, individuals may have a different number of copies of the repeat at a particular locus.

As used herein the term "polymorphism" with respect to a particular  
15 region of a DNA molecule includes naturally occurring variations in nucleotide sequence among individuals that occur in a particular region. Such polymorphisms can occur, e.g., when DNA from one individual has an insertion of an additional nucleotide(s), a deletion of a nucleotide(s), a substitution of a nucleotide(s) when  
20 compared to DNA from another individual. Polymorphisms in microsatellite repeats frequently lead to differences in the length of the repeat that can be easily visualized, e.g., by Southern blot analysis of chromosomal DNA fragments using an oligonucleotide probe to visualize the size DNA fragment containing the particular polymorphic element.

As used herein, the term "SNP" (single nucleotide polymorphism)  
25 includes polymorphisms in a single nucleotide, e.g., that occur when a nucleotide is changed, inserted, or deleted.

As used herein, the term "immune cell" includes cells that are of hematopoietic origin and that play a role in the immune response. Immune cells  
30 include lymphocytes, such as B cells and T cells; natural killer cells; myeloid cells, such as monocytes, macrophages, eosinophils, mast cells, basophils, and granulocytes.

As used herein, the term "costimulate" with reference to activated immune cells includes the ability of a costimulatory molecule to provide a second

signal which is not transduced by an activating receptor (a "costimulatory signal") that induces proliferation or effector function. For example, a costimulatory signal can result in cytokine secretion, e.g., in a T cell that has received a T cell-receptor-mediated signal. As used herein the term "costimulatory molecule" includes

5 molecules which are present on antigen presenting cells (e.g., B7-1, B7, B7RP-1 (Yoshinaga et al. 1999. Nature 402:827), B7h (Swallow et al. 1999. Immunity. 11:423) and/or related molecules (e.g., homologs)) that bind to costimulatory receptors (e.g., CD28, CTLA4, ICOS (Hutloff et al. 1999. Nature 397:263), B7h ligand (Swallow et al. 1999. Immunity. 11:423) and/or related molecules) on T cells.

10 As used herein, the phrase "autoimmune disorder or condition" includes immune responses against self antigens. As used herein, the term "immune response" includes T and/or B cell responses, i.e., cellular and/or humoral immune responses.

As used herein, the term "detect" with respect to polymorphic elements

15 includes various methods of analyzing for a polymorphism at a particular site in the genome. The term "detect" includes both "direct detection," such as sequencing, and "indirect detection," using methods such as amplification or hybridization.

## *II. Isolation of Genetic Material*

20 The subject polymorphic elements are useful as markers, e.g., to identify genetic material as being derived from a particular individual or in making assessments regarding the propensity of an individual to develop a particular disorder or condition, the ability of an individual to respond to a certain course of treatment, or in other diagnostic or prognostic assays described in more detail below.

25 Genetic material suitable for use in such assays can be derived from a variety of sources. For example, nucleic acid molecules (preferably genomic DNA) can be isolated from a cell from a living or deceased individual using standard methods. Cells can be obtained from biological samples, e.g., from tissue samples or from bodily fluid samples that contain cells, such as blood, urine, semen, or saliva.

30 The term "biological sample" is intended to include tissues, cells and biological fluids containing cells which are isolated from a subject, as well as tissues, cells and fluids present within a subject. The subject detection methods of the invention can be used to detect polymorphic elements in DNA in a biological sample in intact cells (e.g.,

using *in situ* hybridization) or in extracted DNA, e.g., using Southern blot hybridization. In one embodiment, immune cells are used to extract genetic material for use in the subject assays.

### 5     *III. Polymorphic Elements In the Costimulatory Receptor Locus*

Any of the PMRs or SNPs identified in the costimulatory receptor locus identified herein (see Tables I, II, and III of the application) can be utilized as a marker to detect DNA polymorphisms among individuals. Several approaches were taken to identify the subject polymorphic elements. In one approach, overlapping  
10     bacterial artificial chromosome (BAC) clones (clones 22700 and 22608) were isolated containing contiguous sequences corresponding to the costimulatory receptors in the order of: CD28, CTLA4, and ICOS. Shotgun sequencing of BAC clones in the region followed by gap closure, sequence alignment and assembly generated 381,403 base pairs of contiguous sequence containing all 3 receptors plus an endogenous  
15     HERV-H type endogenous retrovirus located 366 bp 3' of ICOS in reverse orientation.

A number of PMR sequences were identified in this contiguous sequence. In addition, the ICOS gene locus was localized to this region. In one 181 kb BAC clone containing both CTLA4 and ICOS genomic loci, the ICOS receptor was found to be encoded by 5 exons representing leader sequence, extracellular domain,  
20     transmembrane domain, cytoplasmic domain1 and cytoplasmic domain 2. Polymorphic elements identified in the costimulatory receptor locus (as well as exemplary primers that can be used to amplify them) are set forth in Tables I, II, and III.

In one embodiment, a polymorphic element of the invention is 5' of the  
25     CD28 region. Polymorphic elements residing within nucleotides 243-41772 or the costimulatory receptor locus are 5' of the CD28 region.

In one embodiment a PMR or SNP of the invention is in the CD28 region (e.g., the 5'UT, in an intron, or in the 3' UT region of the CD28 gene) of the costimulatory receptor locus. Polymorphic elements residing within nucleotides  
30     42348 and 73724 are within the CD28 region of the costimulatory receptor locus (see the start and end location of the subject PMR sequences and the location of the SNP sequences in Tables I, II, and III of the specification.) The polymorphic elements

residing within nucleotides 73725 and 203643 are in the intergenic region between CD28 and CTLA4.

In one embodiment, the PMR sequence is in the CD28 gene and is selected from the group consisting of SEQ ID Nos.: 303, 306, 309, 312, 315, and 318 to thereby determine the predisposition of a human subject to develop autoimmune disease.

In one embodiment, the PMR sequence is in the CD28 gene and is selected from the group consisting of SEQ ID Nos.: 3, 6, 9, 12, 15, 18, 21, 24, 27, 30, 33, 36, 39, 42, 44, 48, 51, 54, 57, 60, 63, 66, 69, 72, 75, 78, 81, 84, 87, 90, 93, 96, 99, 102, 105, 108, 111, 114, 117, 120, 123, 126, 129, 132, 135, 138, 141, 144, 147, 150, 153, 156, 159, 162, 165, 168, and 171 to thereby determine the predisposition of a human subject to develop autoimmune disease.

In another embodiment, a polymorphic element of the invention is in the CTLA4 region (e.g., the 5' UT region, in an intron, or in the 3'UT region of the CTLA4 gene) of the costimulatory receptor locus. Preferably, where the polymorphic element is a polymorphic element in the CTLA4 region of the costimulatory receptor locus, the polymorphic element is not in the 3' untranslated region of the CTLA4 gene. In another embodiment, a PMR of the invention is not hR2 and a primer that amplifies a polymorphic element in the CTLA4 region of the costimulatory receptor locus does not amplify an hR2 PMR sequence. PMRs and SNPs residing within nucleotides 203644 and 209793 are within the CTLA4 region of the costimulatory receptor locus (see the start and end location or positions of the subject polymorphic sequences in Tables I, II, and III of the specification.) The polymorphic elements residing within nucleotides 209792 and 272635 are in the intergenic region between CTLA4 and ICOS.

In one embodiment, the PMR sequence is in the CTLA4 gene and is selected from the group consisting of SEQ ID Nos.: 321, 324, 327, 330, 333, 336, 339, 342, 345, 348, 351, 354, and 357 to thereby determine the predisposition of a human subject to develop autoimmune disease.

In one embodiment, the PMR sequence is in the CTLA4 gene and is selected from the group consisting of SEQ ID Nos.: 174, 177, 180, 183, 186, 189, 192, 195, 198, 201, 204, 207, 210, 213, 216, 219, 222, 225, 228, 231, and 234 to

thereby determine the predisposition of a human subject to develop autoimmune disease.

5 In one embodiment, a polymorphic element of the invention is in the ICOS region (e.g., the 5'UT, in an intron, or in the 3' UT region of the ICOS gene) of the costimulatory receptor locus. PMRs or SNPs residing within nucleotides 272636 and 297393 are within the ICOS region of the costimulatory receptor locus (see the start and end location of the subject PMR and SNP sequences in Tables I, II, and III of the specification.)

10 In one embodiment, a polymorphic element of the invention is 3' of the ICOS region. Polymorphic elements residing within nucleotides 300867-380660 are 3' of the ICOS region.

In one embodiment, the PMR sequence is in the ICOS gene locus and is selected from the group consisting of: SEQ ID NO: 360:363, 366, and 369.

15 In one embodiment, the PMR sequence is in the ICOS gene locus and is selected from the group consisting of SEQ ID Nos.: 237, 240, 243, 246, 249, 252, 255, 258, 261, 264, 267, 270, 273, 276, 279, 282, 285, 288, 291, 294, 297, and 300.

#### *IV. Polymorphic Elements In The Costimulatory Receptor Locus And Genetic Diseases*

20 Polymorphisms in the CTLA-4 gene have been linked to various autoimmune diseases, such as insulin-dependent diabetes mellitus (IDDM) (Witas et al., Biomedical Letters 58: 163-168, 1998); Addison's disease, Graves' disease and autoimmune hypothyroidism (Kemp et al., Clin. Endocrinol. 49:609-613, 1998); myasthenia gravis and thymoma (Huang et al., J. Neuorimmunol. 88:192-198, 1998);  
25 lupus (Mehrian et al., Arthritis Rheum. 41:596-602, 1998); thyroiditis, particularly postpartum thyroiditis (Waterman et al., Clin. Endocrinol., 49:251-255, 1998); rheumatoid arthritis (Seidl et al., Tissue Antigens 51:62-66, 1998); Hashimoto's disease (Barbesino et al., J. Clin. Endocrinol. and Metab. 83:1580-1584, 1998); coeliac disease (Djilali-Saiah et al., Gut 43:187-189, 1998); and leprosy (Kaur et al., Hum.  
30 Genet. 100:43-50, 1997). Of these diseases, IDDM, Grave's disease and hypothyroidism (Kotsa, K., et al., (1997). *Clin Endocrinol (Oxf)* 46: 551-4; Marron, M. P., et al., (1997). *Hum Mol Genet* 6: 1275-82) have been found to be associated with certain alleles of the hr2 region of human CTLA-4. The PMR associated with

the hR2 region of CTLA4 has the sequence:

gttgattgcatatatacatatatatatatatatatatat (SEQ ID NO: 546). The PMR associated with the hR1 region of CTLA4 has the sequence: ctctccctt ctccctctct cccttcttct ctctctcttc ctctctt (SEQ ID NO: 547)

5                   Currently, there is no information available on whether the hR2 region confers biologically significant attenuation of CTLA-4 expression or whether this polymorphism is merely a marker for an associated gene closely linked to this CTLA-4 allele. The novel polymorphic elements described herein provide additional markers that may be more closely linked with certain autoimmune disorders or  
10 conditions. As described in the appended Examples, use of the instant polymorphic sequences as markers can provide different results, i.e., different distribution of polymorphisms, than those obtained using the hR2 marker, indicating that the polymorphic elements disclosed herein can be used to further refine genetic alleles linked to the costimulatory receptor locus. Exemplary polymorphic elements of the  
15 invention are shown in Tables I, II, and III.

#### *V. Uses of Polymorphic Elements Of The Invention*

The polymorphic elements of the invention are useful as markers in a variety of different assays. The polymorphic elements of the invention can be used,  
20 e.g., in diagnostic assays, prognostic assays, and in monitoring clinical trials for the purposes of predicting outcomes of possible or ongoing therapeutic approaches. The results of such assays can, e.g., be used to prescribe a prophylactic course of treatment for an individual, to prescribe a course of therapy after onset of a disease or disorder, or to alter an ongoing therapeutic regimen.

25                   Accordingly, one aspect of the present invention relates to diagnostic assays for detecting PMRs or SNPs in a biological sample (e.g., cells, fluid, or tissue) to thereby determine whether an individual is afflicted with a disease or disorder, or is at risk of developing a disorder linked to one or more of the subject polymorphisms. The subject assays can also be used to determine whether an individual is at risk for  
30 passing on the propensity to develop a disease or disorder to an offspring. The invention also provides for prognostic (or predictive) assays for determining whether an individual is at risk of developing an autoimmune disorder or condition. For example, polymorphisms in a PMR or SNP sequence can be assayed in a biological

sample. Such assays can be used for prognostic, diagnostic, or predictive purpose to thereby prophylactically or therapeutically treat an individual prior to or after the onset of an autoimmune disorder associated with one or more polymorphisms.

5 In another embodiment, the methods further involve obtaining a control biological sample from a control subject, determining one or more polymorphic element in the sample and comparing the polymorphisms present in the control sample with those in a test sample.

10 The invention also encompasses kits for detecting the polymorphic elements in a biological sample. For example, the kit can comprise a primer capable of detecting one or more PMR and/or SNP sequences in a biological sample. The kit can further comprise instructions for using the kit to detect PMR and/or SNP sequences in the sample.

Polymorphisms in the costimulatory receptor locus among individuals can be used to identify genetic material as being derived from a particular individual. 15 For example, minute biological samples can be obtained from an individual and an individual's genomic DNA can be amplified using primers which amplify one or more of the disclosed PMR sequences to obtain a unique pattern of bands. A particular band pattern can be compared with a band pattern in a sample known to have come from a certain individual to determine whether the patterns match. Other exemplary methods 20 for detection are set forth below. Panels of corresponding DNA sequences from individuals can provide unique individual identifications, as each individual will have a unique set of such DNA sequences due to allelic differences.

The subject polymorphic elements can also be used in forensic biology. Forensic biology is a scientific field employing genetic typing of biological evidence 25 found at a crime scene as a means for positively identifying, for example, a perpetrator of a crime. For example, to make such an identification, PCR technology can be used to amplify DNA sequences taken from very small biological samples found at a crime scene. The amplified sequence can then be compared to a standard, thereby allowing identification of the origin of the biological sample.

30 The polymorphic elements described herein can further be used to provide polynucleotide reagents, *e.g.*, probes which can be used in, for example, an *in situ* hybridization technique, to identify a specific tissue, *e.g.*, in cases where a forensic pathologist is presented with a tissue of unknown origin.

## VI. Detection of Polymorphisms

Practical applications of techniques for identifying and detecting polymorphisms relate to many fields including forensic medicine, disease diagnosis and human genome mapping.

DNA polymorphisms can occur, e.g., when one nucleotide sequence comprises at least one of 1) a deletion of one or more nucleotides from a polymorphic sequence; 2) an addition of one or more nucleotides to a polymorphic sequence; 3) a substitution of one or more nucleotides of a polymorphic sequence, or 4) a chromosomal rearrangement of a polymorphic sequence as compared with another sequence. As described herein, there are a large number of assay techniques known in the art which can be used for detecting alterations in a polymorphic sequence.

Repeats associated with specific genetic alleles are commonly used as molecular markers in phenotyping human populations. Microsatellite repeats (simple repetitive elements) are defined as motifs of 1-6 bases in length and tandemly reiterated 5-100 times or more. The assay of repeats is amenable to automation, and thus has gained wide use in forensic science and genetic disease linkage determination. These repeats are dispersed throughout the genome and currently are not known to have any definitive biological function, although some reports suggest a role of microsatellites in binding nuclear proteins. Indeed a growing number of genetic diseases are being attributed to the presence of alleles containing unusually large repeats (Epplen, C., *et al.*, (1997). *Electrophoresis* 18: 1577-85).

Analysis of polymorphisms is amenable to highly sensitive PCR approaches using specific primers flanking the repetitive sequence of interest. In one embodiment, detection of the alteration involves the use of a probe/primer in a polymerase chain reaction (PCR) (see, *e.g.*, U.S. Patent Nos. 4,683,195 and 4,683,202), such as anchor PCR or RACE PCR, or, alternatively, in a ligation chain reaction (LCR) (see, *e.g.*, Landegran *et al.* (1988) *Science* 241:1077-1080; and Nakazawa *et al.* (1994) *PNAS* 91:360-364), the latter of which can be particularly useful for detecting polymorphisms in the PMR sequence (see Abravaya *et al.* (1995) *Nucleic Acids Res.* 23:675-682).

This method can include the steps of collecting a sample of cells from a patient, isolating nucleic acid (*e.g.*, genomic, DNA) from the cells of the sample,



contacting the nucleic acid sample with one or more primers which specifically amplify a PMR sequence under conditions such that hybridization and amplification of the PMR sequence (if present) occurs, and detecting the presence or absence of an amplification product, or detecting the size of the amplification product and  
5 comparing the length to a control sample. It is anticipated that PCR and/or LCR may be desirable to use as a preliminary amplification step in conjunction with any of the techniques used for detecting polymorphisms described herein.

Alternative amplification methods include: self sustained sequence replication (Guatelli, J.C. *et al.*, 1990, Proc. Natl. Acad. Sci. USA 87:1874-1878),  
10 transcriptional amplification system (Kwoh, D.Y. *et al.*, 1989, Proc. Natl. Acad. Sci. USA 86:1173-1177), Q-Beta Replicase (Lizardi, P.M. *et al.*, 1988, Bio/Technology 6:1197), or any other nucleic acid amplification method, followed by the detection of the amplified molecules using techniques well known to those of skill in the art. These detection schemes are especially useful for the detection of nucleic acid  
15 molecules if such molecules are present in very low numbers.

In one embodiment, after extraction of genomic DNA, amplification is performed using standard PCR methods, followed by molecular size analysis of the amplified product (Tautz, 1993; Vogel, 1997). Typically DNA amplification products are labeled by the incorporation of radiolabelled nucleotides or phosphate end groups  
20 followed by fractionation on sequencing gels alongside standard dideoxy DNA sequencing ladders. By autoradiography, the size of the repeated sequence can be visualized and detected heterogeneity in alleles recorded. More recent innovations include the incorporation of fluorescently labeled nucleotides in PCR reactions followed by automated sequencing. Both methods have been used in the study of a  
25 human CTLA-4 repeats (Yanagawa, T., *et al.*, (1995). *J Clin Endocrinol Metab* 80: 41-5 Huang, D., *et al.*, (1998). *J Neuroimmunol* 88: 192-8.

In other embodiments, polymorphisms can be identified by hybridizing a sample and control nucleic acids to high density arrays containing hundreds or thousands of oligonucleotides probes (Cronin, M.T. *et al.* (1996) *Human Mutation* 7:  
30 244-255; Kozal, M.J. *et al.* (1996) *Nature Medicine* 2: 753-759). For example, polymorphisms can be identified in two dimensional arrays containing light-generated DNA probes as described in Cronin, M.T. *et al. supra*. Briefly, a first hybridization array of probes can be used to scan through long stretches of DNA in a sample and

control to identify base changes between the sequences by making linear arrays of sequential overlapping probes. This step allows the identification of polymorphisms. This step is followed by a second hybridization array that allows the characterization of specific polymorphisms by using smaller, specialized probe arrays complementary to all polymorphisms detected.

At the present time in this art, the most accurate and informative way to compare DNA segments requires a method which provides the complete nucleotide sequence for each DNA segment. Particular techniques have been developed for determining actual sequences in order to study polymorphism in human genes. See, for example, Proc. Natl. Acad. Sci. U.S.A. 85, 544-548 (1988) and Nature 330, 384-386 (1987); Maxim and Gilbert. 1977. *PNAS* 74:560; Sanger 1977. *PNAS* 74:5463. In addition, any of a variety of automated sequencing procedures can be utilized when performing the diagnostic assays ((1995) *Biotechniques* 19:448), including sequencing by mass spectrometry (see, e.g., PCT International Publication No. WO 94/16101; Cohen *et al.* (1996) *Adv. Chromatogr.* 36:127-162; and Griffin *et al.* (1993) *Appl. Biochem. Biotechnol.* 38:147-159).

In genetic mapping, the most frequently used screening for DNA polymorphisms arising from mutations consist of digesting the DNA strand with restriction endonucleases and analyzing the resulting fragments by means of Southern blots. See Am. J. Hum. Genet. 32, 314-331 (1980) or Sci. Am. 258, 40-48 (1988). Since polymorphisms often occur randomly they may affect the recognition sequence of the endonuclease and preclude the enzymatic cleavage at that site.

Restriction fragment length polymorphism mappings (RFLPS) are based on changes at a restriction enzyme site. In one embodiment, polymorphisms from a sample cell can be identified by alterations in restriction enzyme cleavage patterns. For example, sample and control DNA is isolated, amplified (optionally), digested with one or more restriction endonucleases, and fragment length sizes are determined by gel electrophoresis and compared. Moreover, the use of sequence specific ribozymes (see, for example, U.S. Patent No. 5,498,531) can be used to score for the presence of a specific ribozyme cleavage site.

Another technique for detecting specific polymorphisms in particular DNA segment involves hybridizing DNA segments which are being analyzed (target DNA) with a complimentary, labeled oligonucleotide probe. See Nucl. Acids Res. 9,

879-894 (1981). Since DNA duplexes containing even a single base pair mismatch exhibit high thermal instability, the differential melting temperature can be used to distinguish target DNAs that are perfectly complimentary to the probe from target DNAs that only differ by a single nucleotide. This method has been adapted to detect the presence or absence of a specific restriction site, U.S. Pat. No. 4,683,194. The method involves using an end-labeled oligonucleotide probe spanning a restriction site which is hybridized to a target DNA. The hybridized duplex of DNA is then incubated with the restriction enzyme appropriate for that site. Reformed restriction sites will be cleaved by digestion in the pair of duplexes between the probe and target by using the restriction endonuclease. The specific restriction site is present in the target DNA if shortened probe molecules are detected.

Other methods for detecting polymorphisms in nucleic acid sequences include methods in which protection from cleavage agents is used to detect mismatched bases in RNA/RNA or RNA/DNA heteroduplexes (Myers *et al.* (1985) *Science* 230:1242). In general, the art technique of "mismatch cleavage" starts by providing heteroduplexes of formed by hybridizing (labeled) RNA or DNA containing the polymorphic sequence with potentially polymorphic RNA or DNA obtained from a tissue sample. The double-stranded duplexes are treated with an agent which cleaves single-stranded regions of the duplex such as which will exist due to basepair mismatches between the control and sample strands. For instance, RNA/DNA duplexes can be treated with RNase and DNA/DNA hybrids treated with S1 nuclease to enzymatically digesting the mismatched regions. In other embodiments, either DNA/DNA or RNA/DNA duplexes can be treated with hydroxylamine or osmium tetroxide and with piperidine in order to digest mismatched regions. After digestion of the mismatched regions, the resulting material is then separated by size on denaturing polyacrylamide gels. See, for example, Cotton *et al.* (1988) *Proc. Natl Acad Sci USA* 85:4397; Saleeba *et al.* (1992) *Methods Enzymol.* 217:286-295. In a preferred embodiment, the control DNA or RNA can be labeled for detection.

In still another embodiment, the mismatch cleavage reaction employs one or more proteins that recognize mismatched base pairs in double-stranded DNA (so called "DNA mismatch repair" enzymes) in defined systems for detecting and mapping polymorphisms obtained from samples of cells. For example, the mutY enzyme of *E. coli* cleaves A at G/A mismatches and the thymidine DNA glycosylase

from HeLa cells cleaves T at G/T mismatches (Hsu *et al.* (1994) *Carcinogenesis* 15:1657-1662). According to an exemplary embodiment, a probe based on a polymorphic sequence is hybridized to a DNA molecule from a test cell(s). The duplex is treated with a DNA mismatch repair enzyme, and the cleavage products, if any, can be detected from electrophoresis protocols or the like. See, for example, U.S. Patent No. 5,459,039.

In other embodiments, alterations in electrophoretic mobility will be used to identify polymorphisms. For example, single strand conformation polymorphism (SSCP) may be used to detect differences in electrophoretic mobility between mutant and wild type nucleic acids (Orita *et al.* (1989) *Proc Natl. Acad. Sci USA*: 86:2766, see also Cotton (1993) *Mutat Res* 285:125-144; and Hayashi (1992) *Genet Anal Tech Appl* 9:73-79). Single-stranded DNA fragments of sample and control PMR nucleic acids will be denatured and allowed to renature. The secondary structure of single-stranded nucleic acids varies according to sequence, the resulting alteration in electrophoretic mobility enables the detection of even a single base change. The DNA fragments may be labeled or detected with labeled probes. The sensitivity of the assay may be enhanced by using RNA (rather than DNA), in which the secondary structure is more sensitive to a change in sequence. In a preferred embodiment, the subject method utilizes heteroduplex analysis to separate double stranded heteroduplex molecules on the basis of changes in electrophoretic mobility (Keen *et al.* (1991) *Trends Genet* 7:5).

In yet another embodiment, the movement of nucleic acid molecule comprising polymorphic sequences in polyacrylamide gels containing a gradient of denaturant is assayed using denaturing gradient gel electrophoresis (DGGE) (Myers *et al.* (1985) *Nature* 313:495). When DGGE is used as the method of analysis, DNA can be modified to insure that it does not completely denature, for example by adding a GC clamp of approximately 40 bp of high-melting GC-rich DNA by PCR. In a further embodiment, a temperature gradient is used in place of a denaturing gradient to identify differences in the mobility of control and sample DNA (Rosenbaum and Reissner (1987) *Biophys Chem* 265:12753).

Examples of other techniques for detecting polymorphisms include, but are not limited to, selective oligonucleotide hybridization, selective amplification, or selective primer extension. For example, oligonucleotide primers may be prepared in

which the polymorphic region is placed centrally and then hybridized to target DNA under conditions which permit hybridization only if a perfect match is found (Saiki *et al.* (1986) *Nature* 324:163); Saiki *et al.* (1989) *Proc. Natl Acad. Sci USA* 86:6230).

Such allele specific oligonucleotides are hybridized to PCR amplified target DNA or a number of different polymorphisms when the oligonucleotides are attached to the hybridizing membrane and hybridized with labeled target DNA.

Alternatively, allele specific amplification technology which depends on selective PCR amplification may be used in conjunction with the instant invention.

Oligonucleotides used as primers for specific amplification may carry the polymorphism of interest in the center of the molecule (so that amplification depends on differential hybridization) (Gibbs *et al.* (1989) *Nucleic Acids Res.* 17:2437-2448) or at the extreme 3' end of one primer where, under appropriate conditions, mismatch can prevent, or reduce polymerase extension (Prossner (1993) *Tibtech* 11:238). In

addition it may be desirable to introduce a novel restriction site in the region of the polymorphic region to create cleavage-based detection (Gasparini *et al.* (1992) *Mol. Cell Probes* 6:1). It is anticipated that in certain embodiments amplification may also be performed using Taq ligase for amplification (Barany (1991) *Proc. Natl. Acad. Sci USA* 88:189). In such cases, ligation will occur only if there is a perfect match at the 3' end of the 5' sequence making it possible to detect the presence of a known polymorphism at a specific site by looking for the presence or absence of amplification.

Another process for studying differences in DNA structure is the primer extension process which consists of hybridizing a labeled oligonucleotide primer to a template RNA or DNA and then using a DNA polymerase and deoxynucleoside triphosphates to extend the primer to the 5' end of the template. Resolution of the labeled primer extension product is then done by fractionating on the basis of size, e.g., by electrophoresis via a denaturing polyacrylamide gel. This process is often used to compare homologous DNA segments and to detect differences due to nucleotide insertion or deletion. Differences due to nucleotide substitution are not detected since size is the sole criterion used to characterize the primer extension product.

Another process exploits the fact that the incorporation of some nucleotide analogs into DNA causes an incremental shift of mobility when the DNA

is subjected to a size fractionation process, such as electrophoresis. Nucleotide analogs can be used to identify changes since they can cause an electrophoretic mobility shift. See, U.S. Pat. No. 4,879,214.

5 The use of certain nucleotide repeat polymorphisms for identifying or comparing DNA segments have been described (e.g., by Weber & May 1989. *Am Hum Genet* 44:388; Litt & Luthy. 1989 *Am Hum Genet* 44:397).

Many other techniques for identifying and detecting polymorphisms are known to those skilled in the art, including those described in "DNA Markers: Protocols, Applications and Overview," G. Caetano-Anolles and P. Gresshoff ed.,  
10 (Wiley-VCH, New York) 1997, which is incorporated herein by reference as if fully set forth.

Since a polymorphic marker and an index locus occur as a "pair", attaching a primer oligonucleotide according to the present invention to one member of the pair, e.g., the polymorphic marker allows PCR amplification of the segment  
15 pair. The amplified DNA segment can then be resolved by electrophoresis and autoradiography. A resulting autoradiograph can then be analyzed for its similarity to another DNA segment by autoradiography. Following the PCR amplification procedure, electrophoretic mobility enhancing DNA analogs may optionally be used to increase the accuracy of the electrophoresis step.

20 In addition, many approaches have also been used to specifically detect SNPs. Such techniques are known in the art and many are described e.g., in *DNA Markers: Protocols, Applications, and Overviews*. 1997. Caetano-Anolles and Gresshoff, Eds. Wiley-VCH, New York, pp199-211 and the references contained therein). For example, in one embodiment, a solid phase approach to detecting  
25 polymorphisms such as SNPs can be used. For example an oligonucleotide ligation assay (OLA) can be used. This assay is based on the ability of DNA ligase to distinguish single nucleotide differences at positions complementary to the termini of co-terminal probing oligonucleotides (see, e.g., Nickerson et al. 1990. *Proc. Natl. Acad. Sci. USA* 87:8923. A modification of this approach, termed coupled  
30 amplification and oligonucleotide ligation (CAL) analysis, has been used for multiplexed genetic typing (see, e.g., Eggerding 1995 *PCR Methods Appl.* 4:337); Eggerding et al. 1995 *Hum. Mutat.* 5:153).

20/229 9965899  
In another embodiment, genetic bit analysis (GBA) can be used to detect a SNP of the invention (see, e.g., Nikiforov et al. 1994. *Nucleic Acids Res.* 22:4167; Nikiforov et al. 1994. *PCR Methods Appl.* 3:285; Nikiforov et al. 1995. *Anal Biochem.* 227:201). In another embodiment, microchip electrophoresis can be used for high-speed SNP detection (see e.g., Schmalzing et al. 2000. *Nucleic Acids Research*, 28). In another embodiment, matrix-assisted laser desorption/ionization time-of-flight mass (MALDI TOF) mass spectrometry can be used to detect SNPs (see, e.g., Stoerker et al. *Nature Biotechnology* 18:1213).

10 In one embodiment of the invention, more than one polymorphism (e.g., more than one PMR, more than one SNP, and/or at least one PMR and at least one SNP) may be detected to enhance the ability of a particular polymorphic profile to be correlated with the presence or absence of a disorder or the propensity to develop a disorder.

15 The methods described herein may be performed, for example, by utilizing pre-packaged diagnostic kits comprising at least one probe/primer nucleic acid or antibody reagent described herein, which may be conveniently used, e.g., in clinical settings to diagnose patients exhibiting symptoms or family history of a disease or illness involving a polymorphic elements. In addition, a readily available commercial service can be used to analyze samples for the polymorphic elements of the invention.

## VII. *Primers for Amplification of Polymorphic Elements*

25 Given the discovery of the instant polymorphic elements, primers can readily be designed to amplify the polymorphic sequences by one of ordinary skill in the art. For example, a PMR or SNP sequence of the invention can be identified in GenBank Accession Numbers AF411059 (BAC 22608), AF411058 (BAC 22700) or AF411057 (BAC 22606) or used for homology searching of another database containing human genomic sequences (e.g., using Blast or another program) and the location of the PMR or SNP sequence and/or flanking sequences can be determined and the appropriate primers identified. For example, using the flanking sequences one of ordinary skill in the art could readily identify a primer for use in amplifying a PMR sequence of the invention.

In another embodiment a primer of the invention amplifies a PMR or SNP in the CD28 region (e.g., the 5'UT, in an intron, or in the 3' UT region of the CD28 gene) of the costimulatory receptor locus.

5 In one embodiment, a first or second primer detects a gene in the CD28 locus and comprises the sequence selected from the group consisting of SEQ ID Nos.: 301, 302, 304, 305, 307, 308, 310, 311, 313, 314, 316, and 317.

10 In another embodiment, a primer of the invention amplifies a PMR or SNP in the CTLA4 region (e.g., the 5' UT region, in an intron, or in the 3'UT region of the CTLA4 gene) of the costimulatory receptor locus. Preferably, where the primer amplifies a PMR in the CTLA4 region of the costimulatory receptor locus, the PMR is not in the 3' untranslated region of the CTLA4 gene. In another embodiment, a PMR primer of the invention that amplifies a PMR in the CTLA4 region of the costimulatory receptor locus does not amplify an hR2 PMR sequence.

15 In one embodiment, a first or second primer detects a gene in the CTLA4 locus and comprises or consists of the sequence selected from the group consisting of SEQ ID Nos.: 319, 320, 322, 323, 325, 326, 328, 329, 331, 332, 334, 335, 337, 338, 340, 341, 343, 344, 346, 347, 349, 350, 352, 353, 355, and 356.

20 In another aspect, the invention is directed to a PCR primer capable of amplifying a PMR sequence in the costimulatory receptor locus of a human subject, wherein the primer comprises or consists of a nucleotide sequence selected from the group consisting of: SEQ ID NO: 301, 302, 304, 305, 307, 308, 310, 311, 313, 314, 316, 317, 319, 320, 322, 323, 325, 326, 328, 329, 331, 332, 334, 335, 337, 338, 340, 341, 343, 344, 346, 347, 349, 350, 352, 353, 355, 356, 358, 359, 361, 362, 364, 365, 367, and 368.

25 In one embodiment, a PMR primer of the invention amplifies a PMR in the ICOS region (e.g., the 5'UT, in an intron, or in the 3' UT region of the ICOS gene) of the costimulatory receptor locus.

30 In one embodiment, a first or second primer detects a gene in the ICOS locus and comprises the sequence selected from the group consisting of SEQ ID Nos.: 358, 359, 361, 362, 364, 365, 367, and 368.

In one embodiment, a primer for amplification of a polymorphic elements is at least about 5-10 base pairs in length. In one embodiment, a primer for amplification of a polymorphic elements is at least about 15-20 base pairs in length. In



one embodiment, a primer for amplification of a polymorphic elements is at least about 20-30 base pairs in length. In one embodiment, a primer for amplification of a polymorphic elements is at least about 30-40 base pairs in length. In one embodiment, a primer for amplification of a polymorphic elements is at least about 40-50 base pairs in length. In one embodiment, a primer for amplification of a polymorphic elements is at least about 50-60 base pairs in length. In one embodiment, a primer for amplification of a polymorphic elements is at least about 60-70 base pairs in length. In one embodiment, a primer for amplification of a polymorphic elements is at least about 70-80 base pairs in length. In one embodiment, a primer for amplification of a polymorphic elements is at least about 80-90 base pairs in length. In one embodiment, a primer for amplification of a polymorphic elements is at least about 90-100 base pairs in length. In one embodiment, a primer for amplification of a polymorphic elements is at least about 100-110 base pairs in length. In one embodiment, a primer for amplification of a polymorphic elements is at least about 110-120 base pairs in length. In one embodiment, a primer for amplification of a polymorphic elements is at least about 120-130 base pairs in length. In one embodiment, a primer for amplification of a polymorphic elements is at least about 130-140 base pairs in length. In one embodiment, a primer for amplification of a polymorphic elements is at least about 140-150 base pairs in length. In one embodiment, a primer for amplification of a polymorphic elements is at least about 150-160 base pairs in length. In one embodiment, a primer for amplification of a polymorphic elements is at least about 160-170 base pairs in length. In one embodiment, a primer for amplification of a polymorphic elements is at least about 170-180 base pairs in length. In one embodiment, a primer for amplification of a polymorphic elements is at least about 180-190 base pairs in length. In one embodiment, a primer for amplification of a polymorphic elements is at least about 190-200 base pairs in length.

In one embodiment, a primer for amplification of a PMR sequence of the invention is located at least about 200 base pairs away from (upstream or downstream of) the PMR sequence to be amplified (i.e., leaving about 200 nucleotides from the end of the primer sequence to the PMR). In another embodiment, a primer for amplification of a PMR sequence of the invention is located at least about 150 base pairs away from (upstream or downstream of) the PMR sequence to be amplified. In another embodiment, a primer for amplification of a PMR sequence of

the invention is located at least about 100 base pairs away from (upstream or downstream of) the PMR sequence to be amplified. In another embodiment, a primer for amplification of a PMR sequence of the invention is located at least about 75 base pairs away from (upstream or downstream of) the PMR sequence to be amplified. In another embodiment, a primer for amplification of a PMR sequence of the invention is located at least about 50 base pairs away from (upstream or downstream of) the PMR sequence to be amplified. In another embodiment, a primer for amplification of a PMR sequence of the invention is located at least about 25 base pairs away from (upstream or downstream of) the PMR sequence to be amplified. In another embodiment, a primer for amplification of a PMR sequence of the invention is located at least about 10 base pairs away from (upstream or downstream of) the PMR sequence to be amplified. In another embodiment, a primer for amplification of a PMR sequence of the invention is located at least about 5 base pairs away from (upstream or downstream of) the PMR sequence to be amplified. In yet another embodiment a primer for amplification of a PMR sequence of the invention is adjacent to the PMR sequence to be amplified.

Preferred primers for amplification of a PMR sequence of the invention include the SARA primer pairs set forth in Table II of the specification.

In one embodiment, a primer for the amplification of a PMR sequence comprises a nucleotide sequence selected from the group consisting of: SARA 41, SARA 42, SARA 43, SARA 44, SARA 45, SARA 46, SARA 17, SARA 18, SARA 19, SARA 20, SARA 25, SARA 26, SARA 1, SARA 2, SARA 3, SARA 4, SARA 39, SARA 40, SARA 33, SARA 34, SARA 35, SARA 36, SARA 37, SARA 38, SARA 11, SARA 12, SARA 13, SARA 14, SARA 21, SARA 22, SARA 23, SARA 24, SARA 9, SARA 10, SARA 31, SARA 32, SARA 5, SARA 6, SARA 7, SARA 8, SARA 27, SARA 28, SARA 29, SARA 30, SARA 47, and SARA 48.

In one embodiment, SARA 43 primer is not used to detect a PMR of the invention. In another embodiment, when a SARA 43 primer is used to detect a PMR, it is used in combination with a primer detecting a second, different PMR.

In one embodiment, more than one PMR can be detected, e.g., in a multiplex assay. For example, two sets of primer pairs are used to detect two PMRs. Preferably, when more than one PMR is detected, the PMRs are about 50 kb in distance from each other. For instance, in one example, the SARA primer pairs 47

and 48 are used to detect a first PMR and the SARA primer pairs 1 and 2 are used to detect a second PMR. In another embodiment three different sets of primer pairs are used to detect three PMRs. In yet another embodiment, four different sets of primer pairs are used to detect four PMRs. For example, the SARA primer pairs 31 and 30, 1  
5 and 2, 43 and 44, and 47 and 48 are used in combination to detect four PMRs.

### **VIII. Detecting differentially transcribed genes in genomic DNA**

The instant invention also provides methods of detecting differential transcription of genes in genomic DNA samples. According to the methods, genomic  
10 DNA is subcloned using methods and vectors known in the art, e.g., BAC vectors. Genomic DNA is used to make arrays. Methods of making genomic DNA arrays are known in the art and can be found, e.g., in Lashkari et al. 1997. *PNAS* 94:13057; DeRisi et al. 1997. *Science*. 278:680; Ramsay 1998 *Nature Biotechnology* 16: 40; Wodicka et al. 1997. *Nature Biotechnology* 15:1359; Marshall and Hodgson. 1998.  
15 *Nature Biotechnology* 16: 27; Shoemaker et al. *Nature*. 2001. 409:922 and US Patent 5,807,222. The prior art methods of generating genomic microarrays have relied on finding open reading frames and amplifying them. However, there can be mistakes in computer generated open reading frames. In the instant invention, rather than selecting open reading frames for amplification, randomly picked vectors are used as templates  
20 for amplification, e.g., by PCR, using standard methods such as M13 primers. Thus, the arrays of the instant invention are not based on selecting open reading frames prior to making the arrays. The products of PCR amplification are analyzed for the presence of a single band and are purified using standard methods. PCR products are arrayed onto a solid surface, e.g., slides.

25 Arrays can then be probed using standard methods, for example, total RNA can be prepared from stimulated or unstimulated cells. Probes can be prepared by including a label, e.g., dCTP in a cDNA synthesis reaction.

Hybridization can be performed under standard conditions, e.g., at 42°C for 16h in a buffer containing 50% formamide, 5XSSC, 0.1% SDS and DNA,  
30 e.g., salmon sperm DNA or human COT-1 DNA. The arrays can be washed using standard methods, e.g., in 1X SSC, 0.2% SDS for 5 min, and twice in 0.1X SSC, 0.2% SDS for 10 min and then rinsed in water and dried.

Scanning can be carried out using a commercially available system and the data quantitated.

Using the disclosed methods or variations thereof it is possible to determine not only those genes that are differentially transcribed, but the relative position of the genes in the genome. In one embodiment, this information can be used in a transcription profiling method that examines the correlation between expression patterns of transcribed DNA and loci attributed to genetic diseases. Using such a method, when a disease has been shown to be linked to a particular marker, but it is not known exactly what gene is responsible for the disease, differential regulation of genes in the region of the marker can be examined. In another embodiment, RNA isolated from disease and control samples can be used as probes to determine whether altered transcription levels of gene products exist between the disease and control samples. Because the instant genomic arrays contain positional information, in one embodiment, it is possible to experimentally identify genomic regions bordering transcription initiation, intron/exon boundaries and regions downstream of transcriptional response elements located near a gene. In yet another embodiment, the instant methods can be used to uncover novel genes or transcriptional control elements to which genetic associations are mapped.

The contents of all references, pending patent applications and published patents, cited throughout this application are hereby expressly incorporated by reference. Each reference disclosed herein is incorporated by reference herein in its entirety. Any patent application to which this application claims priority is also incorporated by reference herein in its entirety.

Table 1.

[illegible]











[illegible]

[illegible]

[illegible]

aaagtccttagaattggcctgacac agggtta(SEQ ID NO:190)	cttttgaccctcatagtttggtt cag(SEQ ID NO:191)	Start: 220194 Start: 229431	End: 220253 End: 229467	aaataataatattgatatgtggtaagtattattactatagtatat ttttattattt(SEQ ID NO:192) SARA39;SARA39/40
gtggcccccacagaccctatccttc tggttt(SEQ ID NO:193)	aaaattatagccagtggtgctgc aatcc(SEQ ID NO:194)	Start: 229431	End: 229467	gagagaaagagaagcaaaagcagagagagagagagaga(SE Q ID NO:195)
ctgggtgacagagtggaccctg tctgaaa(SEQ ID NO:196)	aaaaactgctgcttgggtcccaac atagac(SEQ ID NO:197)	Start: 230748 Start: 230749	End: 230810 End: 230810	ac acatccccacacaacaacaca(SEQ ID NO:198) SARA33;SARA33/34
cttgagtactcagggtcctcaaggt tatttc(SEQ ID NO:199)	gcctttttatgttttttctatcttttttct (SEQ ID NO:200)	Start: 231617 Start: 231619	End: 231659 End: 231709	agaaaaaaaagagagagagagaaaaacagaaaaaagaataaa aa(SEQ ID NO:201) SARA35;SARA35/36
gagagagaaaaacagaaaaaaga ataaaaag(SEQ ID NO:202)	gaatgctgaaggaaagtattatgg tcactg(SEQ ID NO:203)	Start: 231661 Start: 234817	End: 231709 End: 234857	ccfttttatgttttttctatctttttttctctctctctctcttcttct(SEQ ID NO:204) SARA37;SARA37/38
tgatgagccaattctgtataataa atctg(SEQ ID NO:205)	gattctgtttcccccaagaaccctg actaa(SEQ ID NO:206)	Start: 234821	End: 234844	tctctcttactccctctctctc(SEQ ID NO:207)
ttttgacattaaacatgttcttcttt cta(SEQ ID NO:208)	atgccattacttggtaaaagtga gctaac(SEQ ID NO:209)	Start: 239361	End: 239421	taaatttatcatggtagtttatataataatcttattatttttttaaaattta tttttttta(SEQ ID NO:210)









	1970	1971	1972	1973	1974	1975	1976	1977	1978	1979	1980	1981	1982	1983	1984	1985	1986	1987	1988	1989	1990	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	2014	2015	2016	2017	2018	2019	2020	2021	2022	2023	2024	2025	2026	2027	2028	2029	2030	2031	2032	2033	2034	2035	2036	2037	2038	2039	2040	2041	2042	2043	2044	2045	2046	2047	2048	2049	2050	2051	2052	2053	2054	2055	2056	2057	2058	2059	2060	2061	2062	2063	2064	2065	2066	2067	2068	2069	2070	2071	2072	2073	2074	2075	2076	2077	2078	2079	2080	2081	2082	2083	2084	2085	2086	2087	2088	2089	2090	2091	2092	2093	2094	2095	2096	2097	2098	2099	2100	2101	2102	2103	2104	2105	2106	2107	2108	2109	2110	2111	2112	2113	2114	2115	2116	2117	2118	2119	2120	2121	2122	2123	2124	2125	2126	2127	2128	2129	2130	2131	2132	2133	2134	2135	2136	2137	2138	2139	2140	2141	2142	2143	2144	2145	2146	2147	2148	2149	2150	2151	2152	2153	2154	2155	2156	2157	2158	2159	2160	2161	2162	2163	2164	2165	2166	2167	2168	2169	2170	2171	2172	2173	2174	2175	2176	2177	2178	2179	2180	2181	2182	2183	2184	2185	2186	2187	2188	2189	2190	2191	2192	2193	2194	2195	2196	2197	2198	2199	2200	2201	2202	2203	2204	2205	2206	2207	2208	2209	2210	2211	2212	2213	2214	2215	2216	2217	2218	2219	2220	2221	2222	2223	2224	2225	2226	2227	2228	2229	2230	2231	2232	2233	2234	2235	2236	2237	2238	2239	2240	2241	2242	2243	2244	2245	2246	2247	2248	2249	2250	2251	2252	2253	2254	2255	2256	2257	2258	2259	2260	2261	2262	2263	2264	2265	2266	2267	2268	2269	2270	2271	2272	2273	2274	2275	2276	2277	2278	2279	2280	2281	2282	2283	2284	2285	2286	2287	2288	2289	2290	2291	2292	2293	2294	2295	2296	2297	2298	2299	2300	2301	2302	2303	2304	2305	2306	2307	2308	2309	2310	2311	2312	2313	2314	2315	2316	2317	2318	2319	2320	2321	2322	2323	2324	2325	2326	2327	2328	2329	2330	2331	2332	2333	2334	2335	2336	2337	2338	2339	2340	2341	2342	2343	2344	2345	2346	2347	2348	2349	2350	2351	2352	2353	2354	2355	2356	2357	2358	2359	2360	2361	2362	2363	2364	2365	2366	2367	2368	2369	2370	2371	2372	2373	2374	2375	2376	2377	2378	2379	2380	2381	2382	2383	2384	2385	2386	2387	2388	2389	2390	2391	2392	2393	2394	2395	2396	2397	2398	2399	2400	2401	2402	2403	2404	2405	2406	2407	2408	2409	2410	2411	2412	2413	2414	2415	2416	2417	2418	2419	2420	2421	2422	2
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aatgatattgtcattttactctaaat tat(SEQ ID NO:280)	atcttagtaacaactgtagtcata ttgtt(SEQ ID NO:281)	Start: 361537	End: 361595	gcttctcctccatcatctttttcactttttccctctctctctctctctttt ttcttt(SEQ ID NO:282)
aattttiaacaactgggatcttctgg gcaac(SEQ ID NO:283)	agatggagtcactctgtctatcca ggctg(SEQ ID NO:284)	Start: 363349	End: 363386	attttttgtttgtttgtttttatctctgtttctttttg(SEQ ID NO:285)
atgcacatgtaccctagaaattttaa gtatt(SEQ ID NO:286)	ctgcgtgttttccaaagagggtgcac cattt(SEQ ID NO:287)	Start: 372334	End: 372378	aaaaaagaaaaagaaaaagaaaaagaaaaagaaaaagaaaa gaaa(SEQ ID NO:288)
tttttattgttgggataggctattc aagt(SEQ ID NO:289)	gaagaaaagaccaacacagacac tctgaaaa(SEQ ID NO:290)	Start: 374670	End: 374720	aaggagacagaaaaaggaggaggaggagggaaaaatgggggaga aggagaaaggag(SEQ ID NO:291)
ccagccgggacacacagtgcgag actccate(SEQ ID NO:292)	ccccactgtactagagcaatcata aggact(SEQ ID NO:293)	Start: 377126	End: 377161	caaaaacaaaaaaacaaaaacaaaaacaaaaa(SEQ ID NO:294)
attccattttgttttctctaaggac act(SEQ ID NO:295)	atgtatctgtggcttatcccgacttc taga(SEQ ID NO:296)	Start: 377473	End: 377523	ttgt gtgtg(SEQ ID NO:297)
atgcacagagacagcactatgtttat ccaag(SEQ ID NO:298)	gagacggagctgtgtctgtgtcccc caggct(SEQ ID NO:299)	Start: 379698	End: 379729	tttttctttcttttcttttcttttttttttt(SEQ ID NO:300)

Table II.

SARA PRIMER PAIRS		Start	End	PMR SEQUENCE
SARA 41 GCTGGCTG GATGACTTG ACC (SEQ ID NO: 301)	SARA 42 CCACTGCA CTCCAGCCT GGG (SEQ ID NO:302)	125017	125041	tatatatatacatat atatatataat (SEQ ID NO: 303)
SARA 43 TATTTCTCC TCTTTCACT GG (SEQ ID NO:304)	SARA 44 TGACCTGAA ATAACATA GA (SEQ ID NO:305)	125845	125892	gtgtgtgtgtgtgt gtgtgtgtgtgtgt gtgtgtgtgtgtgt gtgtgtgtgtgtgt (SEQ ID NO:306)
SARA 45 GGGGGGAC AGGCAAAT GACG (SEQ ID NO:307)	SARA 46 TATTCCAGC ATATTTTTC CA (SEQ ID NO:308)	143199	143252	gaaggaggaaga gaggcagagaga gagaaagggaga gagatggggaga gagaga (SEQ ID NO:309)
SARA 17 GAGACAGT ACAATGGTG TTG (SEQ ID NO:310)	SARA 18 ATGTAAAAA CATAAATAT GTATGTG (SEQ ID NO:311)	146984	147075	gtgtgtgtgtgtac atattgtacaggta ggtattacatatgt atacatattacacg tacagttaatatata tgtgtatgtatgtgt gtacac (SEQ ID NO:312)
SARA 19 TGATTATAC CTAAGAAAT GG (SEQ ID NO:313)	SARA 20 CCACTACAC TCTAGTCTG GG (SEQ ID NO:314)	150056	150091	ttctctctctctct ctctctctctctct ttt (SEQ ID NO:315)
SARA 25 TTTCTGGGT TTTAGATTT GG (SEQ ID NO:316)	SARA 26 TGATAAATA TATTAACCC AG (SEQ ID NO:317)	189057	189081	atatatatatatata tatatatata (SEQ ID NO:318)
SARA 1 CATGCGGG TTAATACT TAAT (SEQ ID NO:319)	SARA 2 TTCTCTAGA GGGACAGA ACG (SEQ ID NO:320)	217444	217492	tctatctatctatct atctatctatctatc tatctatctatctat ccat (SEQ ID NO:321)

SARA 3 TTTCCTGTG CATAGATTT AC (SEQ ID NO:322)	SARA 4 GTTGCACTC CAGCCTGG GCG (SEQ ID NO:323)	219183	219214	gttttgtttgtttgtt tgtctgtttgtttt (SEQ ID NO:324)
SARA 39 CTGGATTG CAGCAGCC ACT (SEQ ID NO:325)	SARA 40 GTGGCCCC ACAGACCCT ATC (SEQ ID NO:326)	229431	229467	gagagaaagaga agcaaagcagag agagagagagag a (SEQ ID NO:327)
SARA 33 ACAGAGTG AGACCCTGT CTG (SEQ ID NO:328)	SARA 34 TGTTGGGA CCCAAGCA GCAG (SEQ ID NO:329)	230749	230810	cacacacacaca cacacacacaca cacacatacacac acacacatcccca cacaacaacaca (SEQ ID NO:330)
SARA 35 CAGGTGCTT CAAGGTTAT TC (SEQ ID NO:331)	SARA 36 AATACTTTC CTTCAGCAT TC (SEQ ID NO:332)	231619	231709	aaaaaaaaaaga gagagagaaaac agaaaaagaata aaaagccttttat gttttctatcttttt ctctcttctctct gtttct (SEQ ID NO:333)
SARA 37 AAGTGTATG AGCCAATTC TG (SEQ ID NO:334)	SARA 38 TTATATCCA TGTATTAGT CA (SEQ ID NO:335)	234817	234857	tctgtctctcttta ctccctctctctcg attctgtttccc (SEQ ID NO:336)
SARA 11 GGTCCTATG TGGTATGAA GG (SEQ ID NO:337)	SARA 12 AGACACAAA ATTACGCAT GC (SEQ ID NO:338)	243340	243365	tatatgtaagtgtgt gtatagatatg (SEQ ID NO:339)
SARA 13 CTTTTCAAA TCTCTGCAT GG (SEQ ID NO:340)	SARA 14 ATGCCTGC CTGGAAAG CTGC (SEQ ID NO:341)	245299	245342	acacacacacac acacacgcacac acacacgcacac acacacac (SEQ ID NO:342)
SARA 21 TGTCTCCCT AACACACTA GG (SEQ ID NO:343)	SARA 22 AATAAAACA GAAACAATA CC (SEQ ID NO:344)	249355	249387	tatatatctatatgt agatctatatctgt ctct (SEQ ID NO:345)

SARA 23 TGCATTTCT TCTCACAGT CC (SEQ ID NO:346)	SARA 24 GTGAAAGG GAGCAGAG AAAG (SEQ ID NO:347)	249821	249860	ctttctctctctc ctttactttatgtt tcctct (SEQ ID NO:348)
SARA 9 TTCTATGCC TCTCTTCTT GG (SEQ ID NO:349)	SARA 10 ATCTAATAT GACAGGTG TCC (SEQ ID NO:350)	253000	253044	tctctctgtgtgt cacatgatctct ctgtgtgtgtgt gt (SEQ ID NO:351)
SARA 31 TGCACTCCA GCCTGAGC GAC (SEQ ID NO:352)	SARA 32 TTCAACACT TAAGAATGG GG (SEQ ID NO:353)	263177	263211	attttattttat ttttattttat (SEQ ID NO:354)
SARA 5 GGTAAGTG ACAGAGTCA GGT (SEQ ID NO:355)	SARA 6 AAAGGATGA CACTCAATT GG (SEQ ID NO:356)	265833	265858	tatatatatatat gtatgtatga (SEQ ID NO:357)
SARA 7 TAGCGGCA ATGTACAGC TGA (SEQ ID NO:358)	SARA 8 CTTCTCTAC AGTTTATAA CC (SEQ ID NO:359)	266114	266161	tgtgtgtgtgtgt gtgtgtgtgtgt gtgtgtgtgtgt gtgtg (SEQ ID NO:360)
SARA 27 TACGAAGTA GTTTAAAAA TG (SEQ ID NO:361)	SARA 28 CACATAGTC TCTATATAT TG (SEQ ID NO:362)	290719	290745	atatacatatat ataaaatatat (SEQ ID NO:363)
SARA 29 ATAAAGCCC CAGATTTT G (SEQ ID NO:364)	SARA 30 CTGGGGAA CAGAGTAAA CCC (SEQ ID NO:365)	290427	290463	gaaaagaaaaga aaagaaaagaaa gagagagaaaa g (SEQ ID NO:366)
SARA 47 ggtgtgaagcat aaagatg (SEQ ID NO:367)	SARA 48 TCCCCTCTC CATGTCCTT TC (SEQ ID NO:368)	295275	295326	gtgtgtgtgtgag gtgtgtgtgtgt gtgtgcacgtgt gtttgtgtgt (SEQ ID NO:369)

SNP POSITION	SNP and 5' and 3' sequence	SEQ ID NO:
SNP 243	taatttccaaaaaaAacgtaataca	SEQ ID NO: 370
SNP 1080	gatggcactGatgtgtttct	SEQ ID NO: 371
SNP 2128	gactccatctaaaaaaAatatatatata	SEQ ID NO: 372
SNP 6930	agttggcttAacttccttct	SEQ ID NO: 373
SNP 8300	gccctcgattAgaatgagag	SEQ ID NO: 374
SNP 9844	ctaatacataAtttttttgaa	SEQ ID NO: 375
SNP 13809	ggattacaggCgcacaccact	SEQ ID NO: 376
SNP 20590	tttcaacaggTagccttactt	SEQ ID NO: 377
SNP 24893	caccttatgtTgtctattttt	SEQ ID NO: 378
SNP 27842	taattgttatCataattatta	SEQ ID NO: 379
SNP 29938	acaccttattCttcatgtaat	SEQ ID NO: 380
SNP 34307	tgcaactgcaCggaaactgaa	SEQ ID NO: 381
SNP 41872	ttttctttttctttTctttctctct	SEQ ID NO: 382
SNP 49112	tttcaagtcaTtttgaagtaa	SEQ ID NO: 383
SNP 50661	caagaaattaGaaaccagcca	SEQ ID NO: 384
SNP 56652	tgtgcattttTcacatgccc	SEQ ID NO: 385
SNP 57187	tgcataaaagCcttcagtaga	SEQ ID NO: 386
SNP 57226	gagagcccaaCctctctaag	SEQ ID NO: 387
SNP 57377	ttctcttttgTcttatctccc	SEQ ID NO: 388
SNP 57435	ttccccacctCaccccagtc	SEQ ID NO: 389
SNP 57826	ttccctccctCctccacctc	SEQ ID NO: 390
SNP 59532	atcattagttAatagagaaat	SEQ ID NO: 391
SNP 60017	agccacatatTgtatgattct	SEQ ID NO: 392
SNP 108669	tttgggtgttNcgggagtttt	SEQ ID NO: 393
SNP 109938	tttctcttttAaaaaacagat	SEQ ID NO: 394
SNP 110501	gtagtgtggtaaaaAatctaaagac	SEQ ID NO: 395
SNP 110684	gtcgagggtcaccCgtgcactgca	SEQ ID NO: 396
SNP 110719	gttcaaaagccAatcccgtaga	SEQ ID NO: 397
SNP 110739	attttcttagcAcagactttac	SEQ ID NO: 398
SNP 114387	aaaaattaagaCattttgtttt	SEQ ID NO: 399
SNP 120280	ctaaaaatacaaaaaAatagccaggc	SEQ ID NO: 400
SNP 120403	acttcagcctGggcaacagag	SEQ ID NO: 401
SNP 121010	ctgactgggtTatttacaatc	SEQ ID NO: 402
SNP 121990	gtcatctctcAataggatgca	SEQ ID NO: 403

SNP 122033	ggaataaacacCtgattgcttc	SEQ ID NO: 404
SNP 126110	tgaagccaacCcacctggat	SEQ ID NO: 405
SNP 127987	acatcagtgaAggacaacact	SEQ ID NO: 406
SNP 128478	aactttttcaGtgatgcaatg	SEQ ID NO: 407
SNP 132652	accacgcttgGggaagggttt	SEQ ID NO: 408
SNP 133418	ttgtcagcatGcaaatcacca	SEQ ID NO: 409
SNP 133520	agtgcctgggGaactgctttt	SEQ ID NO: 410
SNP 134514	accaacaaatTagggtgagg	SEQ ID NO: 411
SNP 139233	gtgtgtgtGtGtggtgtg	SEQ ID NO: 412
SNP 141328	ttttctcttCtttcctaagc	SEQ ID NO: 413
SNP 143835	ttgagggggaAgtctgggcat	SEQ ID NO: 414
SNP 157313	gcctggctaaTttttgtatt	SEQ ID NO: 415
SNP 173359	agacatccatcCaatggaatc	SEQ ID NO: 416
SNP 173984	tcaaaccttctCtgagcagtc	SEQ ID NO: 417
SNP 174036	agatagtgtAcaaaggaaatga	SEQ ID NO: 418
SNP 179878	aaaaaaacGtgaatgtaaa	SEQ ID NO: 419
SNP 183361	cacctcctctCttgcctgcc	SEQ ID NO: 420
SNP 196994	agggaactgaaAattaatctac	SEQ ID NO: 421
SNP 214586	tgtctctactaaaaaaaaaaaaaaaaaaaaaaaaA	SEQ ID NO: 422
SNP 222851	catgaggtgtTgcacctgtg	SEQ ID NO: 423
SNP 223271	tccatttaagCggcagggttt	SEQ ID NO: 424
SNP 224597	cctgtccaaGaatcagggg	SEQ ID NO: 425
SNP 224679	actggctctaCaatagtcag	SEQ ID NO: 426
SNP 225479	gataacaaacTcactcctgtt	SEQ ID NO: 427
SNP 226412	aaatgtgaaaTtatctcactt	SEQ ID NO: 428
SNP 228418	gagtcaccacA	SEQ ID NO: 429
SNP 228913	tcatctttatTgatgctaata	SEQ ID NO: 430
SNP 229855	attcgcatgcA	SEQ ID NO: 431
SNP 230639	ccagctactcGggagctctgag	SEQ ID NO: 432
SNP 230801	acatccccacaAcaacaacaca	SEQ ID NO: 433
SNP 232195	caaacctgcaTattttgcaca	SEQ ID NO: 434
SNP 232790	gagcttgagaAaggaagcctg	SEQ ID NO: 435
SNP 234071	caggatttacA	SEQ ID NO: 436
SNP 234370	atttgatcaaA	SEQ ID NO: 437
SNP 234431	aaatcagtagTctgaataaag	SEQ ID NO: 438

SNP 234996	tcatgtgtag <b>A</b> tcttttttggg	SEQ ID NO: 439
SNP 235532	gggaggtagg <b>T</b> ctactttgcc	SEQ ID NO: 440
SNP 235612	atctaggttc <b>C</b> cagaggggaa	SEQ ID NO: 441
SNP 235928	agcacaggtt <b>G</b> tattgggact	SEQ ID NO: 442
SNP 236693	gtgaatgcag <b>C</b> ataggaaaga	SEQ ID NO: 443
SNP 236971	gaagaagaga <b>G</b> ttgactaaag	SEQ ID NO: 444
SNP 238558	ttagccaggg <b>T</b> aagaaaaaga	SEQ ID NO: 445
SNP 238903	aaaaaaaa <b>T</b> aaaggaatcc	SEQ ID NO: 446
SNP 239015	ttggtgaagg <b>C</b> tggtagtcca	SEQ ID NO: 447
SNP 239867	aaagaattca <b>G</b> aaaattcataa	SEQ ID NO: 448
SNP 240167	gcttcaacca <b>A</b> taaaaatgtg	SEQ ID NO: 449
SNP 240794	tcacttttgg <b>G</b> gtcatatatt	SEQ ID NO: 450
SNP 240825	ggacaagtgt <b>G</b> tattttcaat	SEQ ID NO: 451
SNP 240956	tttctcttgt <b>G</b> cacaaatcat	SEQ ID NO: 452
SNP 241027	agcaggagca <b>G</b> agataatcta	SEQ ID NO: 453
SNP 241354	cttatctgtgaaaaaaa <b>A</b> tgttacgagc	SEQ ID NO: 454
SNP 241836	tacattcaca <b>C</b> aaaaacatgc	SEQ ID NO: 455
SNP 242422	tagtaggtag <b>G</b> ttgtatatgt	SEQ ID NO: 456
SNP 242602	aaaagtttgg <b>G</b> aggttcattt	SEQ ID NO: 457
SNP 242629	tacctacggg <b>G</b> aaaaatagctt	SEQ ID NO: 458
SNP 242712	taaacttggg <b>G</b> aggtagaac	SEQ ID NO: 459
SNP 243729	cataaaatttc <b>A</b> taacttttta	SEQ ID NO: 460
SNP 243917	tgtatgcaca <b>C</b> ttttgcattt	SEQ ID NO: 461
SNP 244266	tgtaactctg <b>C</b> caatgcctga	SEQ ID NO: 462
SNP 244368	gaaaccatgc <b>A</b> tcatcacctc	SEQ ID NO: 463
SNP 245446	tcacatcagac <b>C</b> aatttgtcca	SEQ ID NO: 464
SNP 245550	taaaaaattt <b>A</b> aaaaaaacc	SEQ ID NO: 465
SNP 249741	cctgggtgtt <b>T</b> tcaataaacc	SEQ ID NO: 466
SNP 250288	taattttatgc <b>C</b> tttgaaaggc	SEQ ID NO: 467
SNP 250513	aaactttttg <b>T</b> octcaaacct	SEQ ID NO: 468
SNP 251979	tttattctaagg <b>G</b> cagtggttc	SEQ ID NO: 469
SNP 252130	tgctctccact <b>G</b> ctgttttaa	SEQ ID NO: 470
SNP 252881	acaacagaaa <b>C</b> ttctcataat	SEQ ID NO: 471
SNP 253030	tgatctctct <b>G</b> tgtgtgtgtg	SEQ ID NO: 472
SNP 253686	caccatctta <b>T</b> tttgtcataat	SEQ ID NO: 473

SNP 256499	cctgtaatctGagcacttttg	SEQ ID NO: 474
SNP 256570	gccaacatggCgaaacccctgt	SEQ ID NO: 475
SNP 256654	ggaggctgagTcatgagaatc	SEQ ID NO: 476
SNP 257276	cataccataTacaacattc	SEQ ID NO: 477
SNP 257431	tttaagggtagActaggctaag	SEQ ID NO: 478
SNP 257568	aatttatactGtagatataga	SEQ ID NO: 479
SNP 258093	taattaaaaattttttTcatcttatta	SEQ ID NO: 480
SNP 259397	tattcacagattttTcttttttaaaa	SEQ ID NO: 481
SNP 259905	ttaaaaaatcGatcagtatct	SEQ ID NO: 482
SNP 260191	ttaaataataTaaagaaccaa	SEQ ID NO: 483
SNP 260961	attgtttccaCcaattttaca	SEQ ID NO: 484
SNP 262674	cagagagtctAagatagaacc	SEQ ID NO: 485
SNP 263521	ttatatgttaAttctttaaaa	SEQ ID NO: 486
SNP 263777	aatcaaaaattccCagtggatat	SEQ ID NO: 487
SNP 263844	tgattttcagGttcatttggc	SEQ ID NO: 488
SNP 264175	gaagcagattAttgggcttag	SEQ ID NO: 489
SNP 264654	tatatatatgTgtacatata	SEQ ID NO: 490
SNP 265508	acagggcccccAccatcacacc	SEQ ID NO: 491
SNP 266067	atcagaagagAtggttacact	SEQ ID NO: 492
SNP 300867	cccttgaaaaaTaaggtaatgt	SEQ ID NO: 493
SNP 301816	ggtcagatagAtctgtagaaa	SEQ ID NO: 494
SNP 302415	ggttgggggcaTggaaaataagg	SEQ ID NO: 495
SNP 302474	ataagagatcGgggcgcagag	SEQ ID NO: 496
SNP 302557	agaagtggtcGggggtttctt	SEQ ID NO: 497
SNP 302614	aaggggttggGgtacttgccc	SEQ ID NO: 498
SNP 302711	aaacatgggtGaatataatcaga	SEQ ID NO: 499
SNP 303540	ttagaagcagGtgttttttag	SEQ ID NO: 500
SNP 304319	caaatatataCttatataata	SEQ ID NO: 501
SNP 304693	ggtggcactgTgtctccccctt	SEQ ID NO: 502
SNP 304871	atgcttttgaCcacctcccac	SEQ ID NO: 503
SNP 305199	gaatctgaccGaatcgacca	SEQ ID NO: 504
SNP 305219	aaaatatggcTggctccttct	SEQ ID NO: 505
SNP 305280	tcttcccatgTtctcacctcc	SEQ ID NO: 506
SNP 305357	ctttttcttccAtgaagtcac	SEQ ID NO: 507
SNP 305715	tattcccgtcCaccttgatga	SEQ ID NO: 508



SNP 306765	tggttaattttTtgaaatcttt	SEQ ID NO: 509
SNP 306910	aattttcattTaaaaaacctt	SEQ ID NO: 510
SNP 307177	aaatttattttActtacagttc	SEQ ID NO: 511
SNP 307617	cacacgttcaCgcttccaatg	SEQ ID NO: 512
SNP 307701	tagcaataaaTattatctact	SEQ ID NO: 513
SNP 308314	actgtgaaatGaagttttgtg	SEQ ID NO: 514
SNP 308532	gttaaatgctGtggtgtctga	SEQ ID NO: 515
SNP 308852	agaaattcaaCtgtccagatt	SEQ ID NO: 516
SNP 309162	tcattctcctCtcttatctcc	SEQ ID NO: 517
SNP 309195	cacactggggAaggctcgaa	SEQ ID NO: 518
SNP 309416	cttgtcatcacCtgagaagctc	SEQ ID NO: 519
SNP 309522	actcctgctaCatccttttag	SEQ ID NO: 520
SNP 309753	gtaaaatctgCttacctaacc	SEQ ID NO: 521
SNP 310253	tcactctaacGtggggactca	SEQ ID NO: 522
SNP 310401	atatgataaaaCttttcttctc	SEQ ID NO: 523
SNP 311249	tgtctctactaaaaAtacaaaaaat	SEQ ID NO: 524
SNP 314397	aagccagtctaAccttttctatg	SEQ ID NO: 525
SNP 316490	ctataaatctcCtagaaggaag	SEQ ID NO: 526
SNP 317398	cctggatacaggGcagatgtgga	SEQ ID NO: 527
SNP 318773	aaaggagatgGtcaataggag	SEQ ID NO: 528
SNP 326432	agacgcattaGgggtttggaac	SEQ ID NO: 529
SNP 332250	tttatttattTattaaattttt	SEQ ID NO: 530
SNP 339563	atgaatgcagTgagaaacacg	SEQ ID NO: 531
SNP 342367	cagctttctgtTgtttgatttc	SEQ ID NO: 532
SNP 343135	agggacttggAaagtcaggct	SEQ ID NO: 533
SNP 349945	tctgtgtgtcGggtctccttt	SEQ ID NO: 534
SNP 350161	ataatcttccaAttgaatctca	SEQ ID NO: 535
SNP 350578	ccacccccaggGtttctcactc	SEQ ID NO: 536
SNP 355440	actttatbocCttgtttaggct	SEQ ID NO: 537
SNP 356996	tttctcttatAccatctgttt	SEQ ID NO: 538
SNP 357054	aaaatatattTatagaaagat	SEQ ID NO: 539
SNP 362429	ttaaaactgcaAtaactccaag	SEQ ID NO: 540
SNP 364707	ttcacattctCttaggtaaaag	SEQ ID NO: 541
SNP 366442	ttcacaaaactTttttaactca	SEQ ID NO: 542
SNP 379229	aaaataacatAcaaggaaaaa	SEQ ID NO: 543

2020-06-06

SEQ ID NO: 544  
SEQ ID NO: 545

attcagccaaaAatttctgcta  
tcaaaaatgaAaaaaccacaga

SNP 380507  
SNP 380660

Table IV.

## Summary of 2q33 Sequence Information

Feature Type	Number	Total Length	Ave Length	Std Dev	Proportion of Analyzed Region
Simple Repeats	353	9604	27	27	2.52%
Complex Repeats	368	60536	151	68	15.87%
Grail ORFs	118	18799	159	130	4.93%
DiCTion ORFs	70	17476	250	110	4.58%
Syntenic Mouse >35 bp	70	8497	121	124	-
Costimulatory Receptors (Transcribed Unit)	3	62285	-	-	16.33%
Other Genes/Pseudogenes/EST	17	15382	-	-	4.03%
Sequence Tagged Sites	22	9241			2.42%

Table V.

Feature Table of the Human Costimulatory Receptor Region of Chromosome 2q33

Receptor	Position Start	Position End	Size	Intron	Size	Gene/EST	Position Start	Position End	Size	Reference	Notes
CD28 5' UTR	42348	42569	222			NADH ubiquinone oxidoreductase homolog	7838	8329	491	AF201077	
CD28 CDS-1	42570	42821	52	CD28 intron 1	19883	EST	74209	74882	473	AA311148	from Jurkat T-cell library
CD28 CDS-2	62505	62861	357	CD28 intron 2	2678	EST	75932	76379	447	N30227	from Melanocyte library
CD28 CDS-3	65540	65664	125	CD28 intron 3	5010	EST	88605	88873	268	AA653852	from schizo brain library
CD28 CDS-4	70675	70803	129			EST	93458	93983	525	AA744591	vicinity of multiple repeat elements
CD28 3' UTR	70804	73724	2921			EST	94424	94744	320	H89084	multiple EST hits
CTLA4 5' UTR	203644	203799	156			EST	95762	96257	495	AV237774	multiple EST hits
CTLA4 CDS-1	203800	203908	109	CTLA4 intron 1	2534	Keratin 18 pseudogene	98655	99173	318	L44301	human thymus library
CTLA4 CDS-2	206443	206790	348	CTLA4 intron 2	444	Nucleophosmin pseudogene	100130	101424	1294	M26325 #NM_000224	multiple stops
CTLA4 CDS-3	207235	207346	112	CTLA4 intron 3	1218	EST homolog	108193	109455	1262	M26697 #NM_006993	multiple stops
CTLA4 CDS-4	208585	208669	105			EST homolog	230319	232134	1815	FS1770, AV474005, AI434725	vicinity of multiple repeat elements
CTLA4 3' UTR	208670	209793	1124			EST homolog	241762	242097	335	AV238656, AI037926, AI905493	possible distant L1 repeat
ICOS 5' UTR	272636	272660	25			EST homolog	253467	253534	67	NT3819, AI801031, AW079941	vicinity of multiple repeat elements
ICOS CDS-1	272661	272718	58	ICOS intron 1	18753	EST	257288	257506	218	Unigene cluster homolog HS 30542	cDNA clusters from multiple tissue sources
ICOS CDS-2	291472	291807	336	ICOS intron 2	685	Endogenous retrovirus	260990	261082	192	AA663871	schizo brain library
ICOS CDS-3	292599	292599	107	ICOS intron 3	1032		267282	269005	1723	AA558770, AA054182, T90825	vicinity of multiple repeat elements
ICOS CDS-4	293632	293716	85	ICOS intron 4	1689		287760	303099	5339	AF139170, PIR A44282	79% identity to some retroviral elements
ICOS CDS-5	295406	295419	14								
ICOS 3' UTR	295420	297393	1974								

## Examples

The following materials and methods were used the Examples :

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BAC clone selection: BAC clones were selected on the basis of positive hybridization to CTLA4, CD28 or ICOS coding sequences (Genome Systems, St. Louis, MO). BAC clone DNA was prepared using Concert Mega Preps BAC protocol followed by restriction endonuclease digestion of 1 ug per sample. Digested samples were electrophoresed in 7% TBE agarose gels followed by electrotransfer onto hybrid membranes. Hybridization was performed against random-primed CTLA4, CD28, or ICOS cDNA probes using 0.4% White Rain Shampoo with Conditioner (Gillette, Boston, MA) at 55°C for 1 hour followed by washing with 1x SSC, 1% SDS and then 0.1x SSC, 1% SDS at 55°C until acceptable background was achieved.

15

BAC clone sequencing: BAC clones were shotgun cloned into pUC18 vectors followed by high throughput sequencing (Lark Technologies, Houston, TX). Briefly, BAC clones were sheared by spray nebulization followed by agarose fractionation and purification of 2-4 Kb and 1-2 Kb fragments. Fragments were blunt end cloned into pUC18 SmaI site and subsequently used to generate BAC subclone libraries. Contig assembly was initially performed with GAP4 (Bonfield, J. K., et al. 1998. *Nucleic Acids Res* 26: 3404) and subsequent manual editing performed using Sequencher (Gene Codes, Ann Arbor, MI). Contig gap closure was performed by primer walk sequencing directly on BAC clones using ABI PRISM Big Dye terminator cycle sequencing chemistry and ABI PRISM 373a sequencer. Final assembly and sequence comparison was performed by alignment with Genbank sequences AC010138 (formerly H\_NH0175H04), AC009965, AF225899, and AF225900.

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Sequence verification: 2q33 sequence assembly was verified by BamHI, EcoRI and HindIII digests of BAC clones 22607, 22608 and 22700 and comparison with predicted restriction digest banding patterns. Although fragments were generated from 28,000 Kb to 7 bp were generated, only those ranging from greater than 2 Kb to less than 12 Kb in size were fractionated sufficiently on 0.7% agarose gels for visual

analysis. The only notable discrepancy was found by the presence of a 7.7 kb BamHI restriction fragment in BAC clone 22608 not predicted by sequence data suggesting a base-miscall leading to the elimination of a BamHI site. The sequence results of BAC clone 22700 were further confirmed by restriction mapping the BAC clone using end-labeled oligonucleotide probes as hybridization probes corresponding to predicted EcoRI or SacI fragments. Blots were exposed to phosphoimage plates and processed using Fujix image plate reader and Image Reader software. Twenty-nine blot hybridizations were performed with complete accuracy to predicted DNA fragments within BAC 22700. As an external verification of contig assembly, dotplot analysis (30 bp window, 90% identity) was performed aligning 2q33 sequence with Celera Genomic Axis GA\_X8WHR7H (Release 25, Celera Genomics, Rockville, MD 20850). Resultant alignment demonstrated co-linearity between the two sequences across 300,000 bp suggesting the correct contig ordering of this genomic region.

Sequence analysis: GCG Wisconsin package 10.0 (GCG, Madison, WI) was used for Blast and FastA database searching. Contigs generated by sequencing were compared to protein databases using TblastN to identify potential coding sequences. After final assembly into one contig, sequences were parsed and Blast searches were performed against Genbank EST and STS databases. Positive EST hits with 80% greater were further blasted against Genbank to determine whether cDNA, Unigene or protein identity could be determined. Complex repeats and open reading frame prediction was performed by GAIL (Genomix, Oak Ridge, TN), and DiCTion (Genetics Institute, Cambridge, MA) under default settings. Alignment of ICOS genomic sequences was performed with GAP with a gap length penalty set to zero. The alignment output was displayed positionally using PlotSimilarity with an analysis window of 100 nucleotides. Dotplot of mouse and human ICOS genomic sequences was performed using GeneWorks (Oxford Molecular Group, Campbell, CA) using a window size of 20 nucleotides and 70 % sequence identity cutoff. Cross species genomic sequence alignment was performed using SIM4 (Florea *et al.* 1998) with an F value = 1.3 and word size = 15. Mouse contigs with homologies greater than 35 nt in length were used in further analysis.

Genomic Microarray Expression Analysis: Plasmid preparations of 864 randomly picked colonies from the BAC 22700 subclone library were used as templates for PCR amplification. PCR amplifications were carried out using modified M13 primers in 100 µl reactions containing 10 mM Tris, 1.5 mM MgCl<sub>2</sub>, 50 mM KCl, 200 mM

5 each dNTP, 200 nM each primer, and 1 unit Taq polymerase (Roche Molecular Biochemicals, Mannheim, Germany). PCR products were analyzed by agarose gel electrophoresis and scored for the presence of a single band resulting in 620/864 subclones yielding a robust single band. PCR products were purified using Millipore MultiScreen-FB filter plates essentially as described by the manufacturer (Millipore, 10 Bedford, Massachusetts). Dried PCR products were resuspended in 5M sodium thiocyanate and spotted in duplicate onto Type VI slides (Molecular Dynamics, Sunnyvale, CA) using a GenII arrayer (Molecular Dynamics, Sunnyvale, CA). Probes were prepared by including Cy3 or Cy5 labeled dCTP (Amersham Pharmacia Biotech, Piscataway, NJ) in oligo-(dT) primed first-strand cDNA synthesis reactions from 10 15 mg total RNA essentially as described (Schena *et al.* 1996). Hybridizations were carried out at 42 °C for 16 hrs in buffer containing 50% formamide, 5X SSC, 0.1% SDS and 100 mg/ml human COT-1 DNA (Life Technologies, Rockville, MD). The arrays were washed at room temperature once in 1X SSC, 0.2% SDS for 5 min, and twice in 0.1X SSC, 0.2% SDS for 10 min then rinsed in water and dried with 20 compressed nitrogen. Scanning was carried out using a ScanArray 5000 confocal laser scanner (GSI Lumonics, Waltham, MA) and quantitated using ArrayVision 4.0 (Imaging Research, Inc, St. Catharines, ON, Canada). Data from replicate spots on three arrays were combined by taking the average of the log transformed ratio. Differential upregulation was defined as 1.5 fold induction in at least 5/6 25 measurements and having a total signal intensity above a background threshold (1,000 for Cy3 + Cy5 on BAC37 reference control.)

Microsatellite Polymorphism Analysis: Human donor placental and peripheral blood DNA were used as amplification templates. Single members of oligonucleotide 30 pairs were end-labelled with gamma-<sup>32</sup>P-ATP using T4 polynucleotide kinase (New England Biolabs, Beverly, MA) followed by purification through G25 spin columns. Fifteen µl PCR reactions were performed using Platinum Taq (Life Technologies) according to manufacturer's protocol using 5 pM of each primer and cycled 30 times

with the parameters: 95°C 1 min. 60 °C 1 min., and 72°C 1 min. Amplified microsatellite DNA was fractionated on Novex QuickPoint Sequencing gels (Invitrogen, Carlsbad, CA). Microsatellite amplification primer pairs used included:

SARA 1: CATGCGGGTT AATACTTAAT (SEQ ID NO:319), SARA2:

- 5 TTCTCTAGAG GGACAGAACG (SEQ ID NO:320); SARA 31: TGCACTCCAG CCTGAGCGAC (SEQ ID NO: 352), SARA 32: TTCAACACTT AAGAATGGGG (SEQ ID NO:353); SARA 43: TATTTCTCCT CTTTCACTGG, TGACCTGAAA TAAACATAGA; Sara 47: GGTGTTGAAG CATAAAGATG (SEQ ID NO: 367), TCCCCTCTCC ATTGCCTTTC (SEQ ID NO:368); CTLA4 3'UTR:
- 10 TAGCCAGTGA TGCTAAAGGT TG (SEQ ID NO: 548), AACATACGTG GCTCTATGCA CA (SEQ ID NO:549; position start: 209,177 position end 209,216) ; ICOS 3'UTR retrovirus: GCAAAGAATA AACATTTGAT ATTCAGC (SEQ ID NO:550), CCCCCCTTTG AATGTAATTT TCCTTTACG (SEQ ID NO:551) and having start and end positions at 297,760 and 303,099, respectively.

#### **Example 1. Physical Mapping, Genomic Sequencing and Assembly of 2q33 Costimulatory Receptor Cluster.**

To determine the degree of overlap and distance between CTLA4, CD28, and ICOS, 6 independent BAC clones were isolated by hybridization to

20 costimulatory receptor cDNA probes. Of the 6 separate BAC clones, two exhibited hybridization with CD28, two with CTLA4, one with ICOS, and one with both CTLA4 and ICOS. Each BAC clone was end-sequenced and PCR primer sets were designed to examine BAC clone overlap. Overlapping PCR sets were detected between BAC clones resulting in a hypothetical map of the costimulatory receptor

25 region clustered in the order of CD28, CTLA4, and ICOS. Three fold shotgun sequencing of clone 22700 library resulted in the generation of 1,151 end reads collapsing into 70 contigs spanning approximately 170 kb. Two fold sequencing of clone 22606 and 22608 library generated 960 sequences collapsing into 107 contigs spanning 130 kb, and 960 sequences collapsing into 111 contigs spanning 107 kb,

30 respectively. Mouse BAC clone 23114 was sequenced two-fold generating 767 end read sequences collapsing into 143 contigs spanning 131 kb. Big-Dye primer sequencing was performed directly on BAC clone DNA using primers designed from the sequences flanking gapped sites to close selected gaps in sequence.



BAC clones were end sequenced and PCR primer sets designed specific to each BAC end. Amplification of each BAC clone with the complete set of PCR primers resulted in amplification patterns corresponding to the genomic organization of the costimulatory receptors. Starting and ending positions based on subsequent sequence data are indicated for each BAC clone (N.D. = Not determined):

BAC 22606 (N.D. -66,887), BAC 22607 (N.D.-167,094), BAC 22701 (74,706-278,563), BAC 22699 (84,599-239,485), BAC 22700 (119,296-300,949), BAC 22608 (233,866-381,403).

When necessary, overlaps to publicly available genomic data were used to position contigs, especially PAC clone p61e2 (Accession #AF225900), bridging the 52,408 bp gap between nt. 66,888 to nt. 119,295. Merging BAC clones with existing sequences resulted in one contiguous sequence of 381,403 bp initiating 42,570 bp upstream of CD28, and ending 85,985 bp downstream of ICOS (Figure 1).

## **Example 2. Genomic organization of 2q33 genes, homologs, STS and ESTs.**

Twenty potential protein coding elements were identified within the 381 kb costimulatory receptor region with sequences exhibiting either identity to or homology with known genes or ESTs (Table IV and Table V): NADH:ubiquinone oxidoreductase homolog, CD28 (NM\_006139), keratin-18 pseudogene, nucleophosmin pseudogene, CTLA4 (NM\_005214), Unigene HS.30542 homolog, ESTs, ICOS (Genseq # V53199), and an element similar to many human endogenous retrovirus type H with associated 5' and 3' LTR (RTLH-H2, M18048; amongst others). Based on a recent mapping study of 2q31-33, the three receptor loci within this region are situated on the chromosome with CD28 being the most centromeric and markers, now known to be near ICOS, being the most telomeric (Deng, Z., et al. 2000. *Am J Hum Genet* 67:737). In addition, 22 STS (sequence tag sites) were identified upon BLAST search of this compiled region of 2q33, of which 4 correlated to endogenous retroviral sequence. The commonly used genetic markers for 2q33, D2S307 (SARA 43), D2S72, D2S105, and 19E07-1 were contained within the sequence presented here. Because HERV-H elements are found in ~1000 copies in the genome, it remains to be determined if these 4 STS are specific for the element described here. Based on human ICOS cDNA sequence data, the organization of the ICOS locus was determined to be comprised of 5 coding sequences spanning 22,758

bp from the initiation codon of exon 1 to the termination codon of exon 5, unlike the 4 exon structure of both the CTLA4 and CD28 genes. ICOS exon 5 encoded the smallest coding sequence, represented by only 4 amino acids [(D)-V-T-L] followed by a stop codon. In other respects, exons 1-4 parallel the genomic organization of CTLA4 and CD28 with exon 1 encoding the leader sequence, exon 2 encoding the extracellular Ig-V like domain, exon 3 encoding the transmembrane domain and exon 4 and 5 encoding the cytoplasmic domain. All three costimulatory receptors shared similar pattern of intron size distribution in which intron 1>intron 3> intron 2. ICOS appeared to be more similar in genomic organization to CD28, with ICOS intron 1 spanning 18.7 kb compared to CD28 intron 1 spanning 19.9 kb, versus CTLA4 intron 1 spanning 2.5 kb.

### Example 3. Computer Assisted Prediction of Open Reading Frames.

The 381 Kb costimulatory receptor locus was analyzed by the open reading frame prediction programs DiCTion and GRAIL to assess the potential of other sequences in this region to encode gene products (Figure 1, Table IV). DiCTion analysis of the costimulatory receptor region resulted in the prediction of 70 ORFs with a cumulative length of 17476 bp, of which 5 ORFs represented repetitive Alu sequences. Coding sequences representing CD28 exon 2 and CTLA4 exon 2, keratin-18 and nucleophosmin pseudogenes were predicted by DiCTion. DiCTion did not predict sequences encoding ICOS. Of the remaining ORFs, two were localized to intron 1 of CD28, and single ORFs were predicted in intron 3 of both CTLA4 and ICOS receptor loci. Assuming that the predicted intronic ORFs are false positives, these results suggest that up to 56 potential DiCTion ORFs remain in this region of 381 kb. GRAIL analysis generated more potential ORFs than DiCTion, with a total of 118 segments and a cumulative length of 18,799 bp (Table IV). GRAIL predicted some open reading frames containing CD28 (CDS-1, CDS-2, CDS-4), CTLA4 (CDS-2), and ICOS (CDS-1, CDS-2, CDS-4), however, neither GRAIL or DiCTion were successful in predicting the complete set of exonic sequences from any receptor and moreover, both programs predicted ORFs in known intronic sequences. For example, in the CD28 intron 1, GRAIL predicted 8 ORFs while DiCTion predicted 1 ORF. Although it has been reported that CD28 may be expressed as alternatively spliced products (Lee *et al.* 1990. *J Immunol* 145: 344-52), it has not been demonstrated that

intronic sequences described here contribute to the final products of known isoform variants. When DiCTion and GRAIL outputs were compared, 13 predicted open reading frames were found in common to both. Of these, three correspond to the known sequences CD28 CDS-2, CTLA4 CDS-2 and EST M26697.

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#### **Example 4. Genomic Microarray Expression Analysis (GMEA).**

To examine whether differentially transcribed genes within this genomic region could be detected, the sequenced BAC 22700 subclone library collection was interrogated by genomic microarray expression analysis. The previously sequenced plasmid library DNA samples were amplified by PCR, the amplified DNA products were spotted onto glass slides, and hybridization was performed with total RNA from either non-stimulated or PMA-ionomycin treated CD4+ T-cells. Of the starting 864 plasmid subclones, 620 amplified products were recovered and analyzed, resulting in 18 clones showing differential hybridization in 5 out of 6 replicate experiments (3 slides each with duplicate spots). Eight clones corresponded to sequences within the CTLA4 locus, 7 clones corresponded only to the ICOS 3' UTR and 3 clones corresponded to both ICOS 3' UTR and endogenous retroviral sequences immediately 3' of ICOS (Figure 2A). It must be noted that hybridization of cDNA against genomic DNA would preferentially occur between target sequences of longer length (exon 2 and 3' UTR of CTLA4 and ICOS); thus the degree of hybridization to microarrayed spots containing only short CDS flanked by non-differentially expressing intronic sequences could be lower. Indeed, the differential hybridization detected to ICOS was to the region corresponding to the longest transcribed unit, the 2 kb 3' UTR. Most importantly, no clones other than CTLA4, ICOS and retrovirus immediately downstream of ICOS were found to be induced suggesting that the stringency of the experimental conditions used in this study was sufficient for detecting transcriptionally induced genes while effectively eliminating non-specific background hybridization generated by genomic and plasmid DNA.

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To determine whether hybridization to ICOS and retroviral sequences reflected transcription from the ICOS promoter or whether this differential signal reflected transcripts from the endogenous retrovirus proximal to the ICOS locus, RNA blots were performed to determine transcript orientation from this region. In

order to rule out cross hybridization to repetitive sequences, blast search was performed using ICOS 3' UTR sequences adjacent to the endogenous retrovirus. No repetitive DNA was detected, and hence, this sequence was subcloned in both orientations into separate T7-promoter bearing vectors to generate strand-specific radiolabeled probes. RNA from two donor CD4+ T-cells and Jurkat T-cell line preparations, cultured either in the presence or the absence of PMA-ionomycin activation, were fractionated, blotted and hybridized to either the ICOS 3' UTR sense or anti-sense probe (Figure 2B). With the ICOS anti-sense probe, a clear hybridization signal was observed for activated samples but not for non-activated samples. Hybridization with ICOS sense probe also revealed two regions of clear hybridization signals in all samples examined; one discrete band at approximately 6.5 kb and one non-discrete band at ~3-4 kb. These results strongly suggest that the retroviral LTR promoters 3' of ICOS are transcriptionally active and are responsive to cell activation. The 6 kb band appeared to be preferentially induced on activated CD4+ T-cells while being constitutively expressed in both Jurkat cells samples. The 3-4 kb band appeared to be expressed in all samples examined regardless of activation state. Because these retroviral transcripts may be derived from either the 5' LTR or the 3' LTR viral promoter, at least two potential sets of transcripts may be detected. With the presence of 8 canonical polyadenylation signals (AATAAA) within the 7.5 kb upstream from the ICOS 3' UTR, it is not possible to correlate promoter activity with observed transcript size at this time.

#### **Example 5. Analysis of Microsatellite Polymorphisms.**

Polymorphisms in the 3' UTR of CTLA4 have been linked to a number of autoimmune genetic diseases. To identify additional markers in this region that may also serve to refine the associations between genetic diseases and the costimulatory receptor region of 2q33, 25 microsatellite repeat sequences in the BAC 22700 clone were analyzed for the presence of repeat unit polymorphisms. Genomic DNA PCR amplification of 13 individuals revealed 4 microsatellites, corresponding to di-, tri- and hexanucleotide repeats, that demonstrated allelic polymorphisms upon analysis by denaturing acrylamide gel electrophoresis (Figure 3). Of the 4 polymorphic microsatellite repeats examined, repeat SARA 31(nt. 263,177-263,211; [ATTTTTT]n6) was represented by 2 alleles, repeat SARA 1(nt. 217,444-217,492;

[TCTA]<sub>n</sub>12) was represented by 4 alleles, while SARA 43 (nt. 125,845-125,892 [GT]<sub>n</sub>24, homologous to sequences within D2S307) and SARA 47 (nt. 295,275-295,326; [GT]<sub>n</sub>15) appeared to be highly polymorphic with at least 6 different alleles within 13 individuals examined. Analysis of the 13 individuals for the polymorphisms associated with the known CTLA4 3' UTR (nt. 209,177-209,216; [AT]<sub>n</sub>40) microsatellite repeat demonstrated 2 alleles. Compilation and comparison of the 4 polymorphic microsatellite alleles found in these individuals revealed no shared allelic combination, indicating that this set of 4 polymorphic markers may be effectively applied to the high resolution discrimination of genetic associations of disease states linked to the costimulatory receptor region. For a positive amplification control, a primer set was used corresponding to nt. 297,362 to 297,388 (forward primer) and 297,934 to 297,907 (reverse primer) corresponding to the 3' UTR of ICOS and to the 3' LTR of the HERV-H. Amplification of the 13 individuals with this set of primers resulted in a single predicted band at ~400 bp indicating the presence of this segment of DNA across the panel examined.

#### **Example 6. Cross species comparison of ICOS.**

The generation of the complete sequence for the human ICOS locus along with the partial sequencing of the mouse ICOS locus allowed the cross species comparison of genomic coding and non-coding sequences in this region (Figure 4A, B, C). Limited gap closure of the mouse ICOS locus by primer walking resulted in the assembly of one contiguous sequence spanning CDS-2 to CDS-5 and flanked by 2265 bp of intron-1 and 1415 bp of 3' untranslated/genomic DNA. Dotplot comparison analysis of the human genomic region was performed with the syntenic genomic region from mouse starting from 2265 bp upstream of mouse CDS-2 to 1414 bp downstream from mouse CDS-5 (Figure 4B). Allowing for gaps, diagonals representing a minimum of 60% sequence identity were clearly observed in this aligned region; most notably, a diagonal was detected extending 3' of CDS-5 for 2.4 Kb. A similarity plot of the gap-corrected sequence alignment of this region resulted in approximately 60% sequence identity over 6.4 kb of aligned sequence. The highest peaks of sequence similarity (~80% identity) were clearly detected for CDS-2, CDS-3, CDS-4 and CDS-5. Intron 2 and intron 3 had lower similarity score (~45%) owing to the presence of gaps formed by the alignment process. Gaps in alignment

represented by valleys (<30% identity) were generally comprised of repetitive sequences presented in only one species. Seven peaks of high sequence identity (>70%) were found in non-coding regions of intron 4 and the 3' UTR region starting from 1 kb upstream to 2.4 kb downstream of CDS-5. The sequence conservation in the ICOS intron-4 was especially striking, as evidenced by the presence of the SARA 47 microsatellite in both mouse and human sequences. The SARA 47 (GT)<sub>n</sub>24 intron 4 microsatellite repeat was located 88 bp 5' of human ICOS exon 5, while a similar (GT)<sub>n</sub>48 intron 4 microsatellite repeat was discovered 66 bp 5' of mouse ICOS exon 5.

Sequences flanking ICOS CDS-1 revealed two zones of high similarity between mouse and human genomic DNA (Figure 4A). The first zone of high sequence identity was a 317 bp region with 72% sequence identity to mouse sequences located 276 bp upstream from initiation methionine at nt 272,661. The second zone was a 269 bp region with 75% sequence identity immediately flanking and including CDS-1, starting from 134 bp upstream of the initiation methionine to 75 bp downstream from the start of intron 1. The intervening gap (human = 143 bp, mouse = 448 bp) between zone 1 and zone 2 was due to a G-deficient tract of DNA unique to mouse sequence and populated with numerous low complexity TCCA, TACA and TTCA repeats. Assuming that transcriptional control regions are conserved between mouse and humans, it is likely that sequences in either zone 1 or zone 2 are responsible for transcriptional control of ICOS expression. The full-length human ICOS cDNA (GenSeq # V53199) reveals 25 bp of 5' UTR prior to initiation codon, however, whether this cDNA clone represents the actual transcription start site remains to be determined. Neither mouse or human ICOS zone 2 contains the conventional TATA promoter motif, suggesting that transcriptional start site is likely to be in zone 1 which contains multiple TATA sites. Analysis for conserved transcription factor binding sites located in both zone 1 and zone 2 by the publicly available Transfac database search revealed no T-cell specific control elements shared between mouse and human sequences. A single potential NFAT-1 site was found in mouse zone 1 along with numerous non-T cell specific sites (e.g. AP-1, AP-2, Pu.1, GATA-1, c-Jun, Gal4 and others).

The extent of sequence conservation within the intergenic region encompassing CTLA4 and ICOS receptors was examined by a comparative genomic

survey of a 2x sequenced syntenic mouse BAC clone comprising 143 non-contiguous sequences aligned to the repeat-masked (DUST) human 381 kb sequence using SIM4.

Of regions greater than 34 bp in length, 71 alignments were found with identity scores averaging 81%. When human sequences between nt 100,000 and 301,000 were examined, repetitive sequences comprised 36,621 bp, leaving a total of 164,379 bp of potential structural or transcribed DNA. Within this region, SIM4 mouse homologies totaled 8,531 bp theoretically corresponding to roughly 5% of the CTLA4/ICOS region. Given the limited degree of mouse BAC clone sequence coverage, only 131 kb of data was generated with the potential for an additional missing 28 kb in

"unfilled" gaps, leaving the sequence determination of the syntenic mouse region be approximately 80% complete. Based on the 5% homology estimated between mouse genomic DNA syntenic and shared with human BAC clone 22700, it is not likely that extensive sequence similarities span the intergenic region between CTLA4 and ICOS, but rather, similarities are comprised of smaller stretches of homologous DNA within this region. It remains to be determined whether these stretches of homologous genomic DNA are involved with transcriptional control or whether they encode other peptide domains common to both species.

## EQUIVALENTS

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.